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**PROCEEDINGS OF THE AMERICAN SOCIETY
OF BIOLOGICAL CHEMISTS.**

NINETEENTH ANNUAL MEETING.

Washington, D. C., December 29-31, 1924.

SOME APPLICATIONS OF THE NEW CYSTEINE REACTION.

By M. X. SULLIVAN.

(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)

The cysteine reaction—a red color with β -naphthoquinone-4-sodium sulfonate in the presence of alkali and a reducing agent such as Na_2SO_3 —can be used to determine cysteine quantitatively in the presence of other amino acids and sulfhydryl compounds. Cystine slowly gives the reaction to a slight degree. If cystine is treated with aqueous NaCN it is reduced to cysteine and then gives the reaction promptly. In strong polyneuritis on a rice diet, the tissues of pigeons have little if any cysteine, but do contain cystine; while on a rice diet plus vitamin, the tissues of the pigeons contain cysteine as well as cystine. In extreme polyneuritis the reducing capacity of the tissues seems to be suspended.

THE QUANTITATIVE STUDY OF THE PHYSIOLOGIC ACTION OF THYROXIN.

By EDWARD C. KENDALL.

(From the Department of Biochemistry, Mayo Foundation, Rochester, Minnesota.)

The isolation of thyroxin in pure crystalline form permitted the fact to be shown that thyroxin alone increases the rate of combustion in the animal organism. Furthermore, this increase is related quantitatively to the amount injected. 1 mg. given to an adult produces an increase of approximately 2.5 per cent. The substance acts in minute amounts for long periods, and produces such enormous increases in the output of carbon dioxide above the former level, that there is no escape from the conclusion that thyroxin acts as a catalyst.

Through a study of closely related compounds, which are synthetically prepared, the fact was demonstrated that thyroxin

can exist in two forms: reduced and oxidized. Thyroxin, as isolated from the gland, is the reduced form.

Alpha-oxy-indole propionic acid, the precursor of thyroxin, acts as a reducing agent. It loses 2 atoms of hydrogen with molecular oxygen when the pyrrole ring in the molecule is open, and forms a bond from the nitrogen to number seven carbon in the benzene ring. This compound has feeble oxidizing power. When, however, the pyrrole ring is closed and the bond is present from the nitrogen to number seven carbon, the oxidizing power of the compound is very much increased. The oxidizing potentials of the open and closed ring compounds, when both exist in their oxidizing form, have been measured, and a difference of at least 0.3 volt was found.

When they are injected into a normal dog, the reduced form and the oxidized but open ring form produce no visible response; the oxidized closed ring form causes a marked physiologic effect. There is a drop in blood pressure, an increase in pulse rate, marked increase in respiration, and an increase in the rate of metabolism.

The function of thyroxin is to furnish a compound that can be acted on by mild oxidizing agents, among which is molecular oxygen, and which can then by an intramolecular rearrangement produce an intensely oxidizing substance.

It is significant that oxidation in the animal organism is accelerated by the presence of an agent which is an active hydrogen acceptor, and the degree of stimulation is dependent on the oxidizing potential of this hydrogen acceptor.

A METHOD FOR THE DETERMINATION OF THE pH OF CEREBROSPINAL FLUID.

By A. T. SHOHL AND IRVINE McQUARRIE.

(*From the Department of Pediatrics, Yale University, New Haven.*)

The spinal fluid is obtained over mercury without exposure to air. The sampling tube is calibrated at 0.1 cc. for the first cc. and at 1 cc. intervals up to 10 cc. A measured amount of phenol red is introduced. The lumbar puncture needle is connected to the receiving tube by a sterilized glass connecting piece and with a short rubber tube. The technique is similar to that used in obtaining blood for measuring the CO₂ content. The sampling

tube is of the same diameter and same thickness as the comparator tubes. Without transfer the pH is read in the sampling tube colorimetrically at 38° by comparing either with Sørensen's phosphate standards at 38° and subtracting 0.03 pH or by means of the bicolorimetric standards of Hastings and Sendroy. The CO₂ content is determined by Van Slyke's method. Using a pK value of 6.20 as determined experimentally and from the literature the tension of CO₂ can be calculated.

DISTRIBUTION OF ELECTROLYTES IN BLOOD.

BY D. D. VAN SLYKE AND A. B. HASTINGS.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The conclusion of Van Slyke, Wu, and McLean¹ has been confirmed that HCO₃ in blood is distributed between cells and serum in such manner that the ratio $[\text{HCO}_3]_{\text{cells}} : [\text{HCO}_3]_{\text{serum}}$ varies with CO₂ tension in agreement with a formula deduced from Donnan's law and the base-binding powers of the cell and serum proteins, the validity of the laws of ideal solutions being assumed as a first approximation. The effect of oxygen tension changes has now been also studied, and found to agree with that predicted from the difference in base-binding power between reduced and oxygenated hemoglobin. The ratio $[\text{Cl}]_{\text{cells}} : [\text{Cl}]_{\text{serum}}$ has likewise been studied, using the nitric acid ashing method for Cl determination.² The absolute values for the Cl ratios are somewhat lower than the HCO₃ ratios, but parallel the latter and show approximately the same changes with varying pH and oxygenation. In order to obtain direct data on ion activity ratios, the ratios $[\text{H}^+]_{\text{cells}} : [\text{H}^+]_{\text{serum}}$ and $[\text{Cl}']_{\text{cells}} : [\text{Cl}']_{\text{serum}}$ were determined electrometrically. The ratios thus determined were found to approach equality, to lie at a lower level than the total [Cl] and [HCO₃] ratios, but to parallel the latter as influenced by pH and oxygen changes.

¹ Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

² Van Slyke, D. D., *J. Biol. Chem.*, 1923-24, lviii, 523.

MOVEMENT OF ELECTRICALLY CHARGED ATOMS INSIDE RED BLOOD CORPUSCLES.

By J. F. McCLENDON.

(From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis.)

Ionic mobility was measured by means of the Wheatstone bridge method for determining electric conductivity of solutions. In order to reduce inductance the resistance wires were perfectly straight and the resistance of the cell, full of erythrocytes washed in sugar solution, was balanced against the resistance of a similar cell filled with a known KCl solution at 25°. Owing to the insulating properties of the surfaces of the erythrocytes, the resistance, R , to direct current is very high. The impedance, Z , to alternating current of low frequency was also high since $Z = \sqrt{X^2 + R^2}$ where X is the reactance due to the insulating membranes and $X = \frac{1}{2\pi fC}$ where f is the frequency and C is the capacitance of the condensers formed by interposition of the insulating membranes. By increasing the frequency the reactance is reduced and when this is reduced to a negligible quantity, the resistance of the cell interiors may be determined. The only difficulty lies in the fact that the volume occupied by the membranes and the sugar solution is unknown. The high resistance of the membranes is illustrated by the fact that if we take the impedance measured with a frequency of 1,000,000 cycles per second as the resistance of the erythrocyte interior, the impedance is increased 400 per cent due to the reactance of the membranes when measured with a frequency of 1,000 cycles per second. Preliminary experiments indicate that the conductivity of the ox erythrocyte interior is about equal to or greater than that of a 0.01 N KCl solution.

PHYSICOCHEMICAL METHODS OF CHARACTERIZING PROTEINS.

VII. THE MOLECULAR WEIGHTS OF THE PROTEINS.

By EDWIN J. COHN.

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

The molecular weights of the proteins constitute their outstanding physical characteristic. Their estimation by such direct methods as the determination of the osmotic pressures they produce has, however, not often² led to consistent results.

Although the molecular weights of most proteins have been in doubt, the minimal molecular weight of hemoglobin has been well known for over 30 years. In 1894 Hüfner found that 16,721 gm. of hemoglobin combined with each mol of carbon monoxide, or with a weight of the protein that contained 1 atom of iron. The simultaneous consideration of analytical and physicochemical information thus led, at an early date, to a very accurate minimal molecular weight for hemoglobin and has subsequently led to estimates of the minimal molecular weights of other proteins.

Solubility and electromotive force measurements offer new physicochemical methods of determining the equivalent combining weights of proteins, and amino acid determinations in protein hydrolysates provide new analytical evidence from which can be calculated the minimal weight of a protein that contains 1 molecule of an amino acid. From such analytical and physicochemical data the minimal molecular weights of fourteen proteins have been deduced.

Given the minimal molecular weights of proteins, their true molecular weights may be estimated by determining the relative size of their molecules. This is being accomplished by dialysis and ultrafiltration through membranes of graded permeability. The probable molecular weights of the proteins are thus obtained (Table I) as integral multiples of their minimal molecular weights.

² The investigations of Sørensen, in which account has been taken of membrane equilibria, constitute the outstanding exceptions. His results are in good agreement with those reported.

TABLE I.

Protein.	Minimal molecular weight.	Probable molecular weight.
Bence-Jones' protein.....	12,250	24,500
Egg albumin.....	33,400	33,400
Serum albumin.....	5,000	45,000
Hemoglobin, horse.....	16,700	66,800
" ox.....	33,400	66,800
Fibrin.....	14,000	42,000*
Serum globulin.....	27,000	81,000*
Hemocyanin, <i>Limulus</i>	22,700	90,800*
Zein.....	19,400	97,000*
Glutenin.....	36,300	108,900*
Edestin.....	29,000	116,000*
Gelatin.....	10,300	123,600*
Gliadin.....	20,700	125,000*
Casein.....	12,800	192,000*

* The estimation of the molecular dimensions of these larger proteins is not yet complete. The true molecular weights may, therefore, be still larger multiples of these minimal molecular weights.

ACETYLATION AS A DETOXICATING REACTION.

By JOSEPH B. MUENZEN, LEOPOLD R. CERECEDO, AND CARL P. SHERWIN.

(From the Chemical Research Laboratory, Fordham University, New York.)

In studying the detoxication of para-aminophenylacetic acid it was found that the human being as well as the rabbit detoxicates the compound by joining acetic acid to the amino group, while the dog detoxicates the foreign molecule by conjugating glycocoll with it, thus forming para-aminophenaceturic acid. A number of amino compounds were investigated in order to determine the influence of various other radicals on acetylation. It was found, contrary to the report of Salkowski, that the human organism is able to acetylate both para- and meta-aminobenzoic acid.

In general we found that the dog will acetylate an aliphatic amino compound or the side chain of an aromatic compound, while the human being and the rabbit may fail to acetylate these compounds entirely, but are able to acetylate the amino groups

attached directly to the benzene ring. Acetylation in the body is apparently limited to amino compounds.

The influence of diet on acetylation was studied, as well as the effect of certain amino acids and certain compounds believed to be products of intermediary catabolism of proteins, fats, and carbohydrates.

Attempts were made to locate the seat of the reaction, and it was found that acetylations in the human being as well as in the rabbit are very likely confined to the liver.

The possibility of using this reaction as a functional liver test was investigated and yielded promising results.

THE RELATION OF HISTIDINE AND ARGININE TO CREATINE AND PURINE METABOLISM.

By WILLIAM C. ROSE AND KENNETH G. COOK.

(*From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.*)

Studies have been made of creatine and purine metabolism in growing rats upon diets in which the nitrogen was supplied, respectively, in the form of casein, completely hydrolyzed casein, and hydrolyzed casein from which histidine and arginine had been precipitated by the Kossel-Kutscher procedure. Despite the inherent difficulties involved in quantitative urine collections in rats, the results of the experiments are quite consistent. The diets of whole casein and of completely hydrolyzed casein led to the excretion of progressively increasing quantities of total creatinine (creatine plus creatinine), allantoin, and uric acid, which were roughly proportional to the increments in body weight of the animals. On diets of hydrolyzed casein from which histidine and arginine had been precipitated, the output of allantoin decreased 40 to 50 per cent. The uric acid excretion also showed a tendency to decrease, but the variations from the normal values were less striking than in the case of allantoin. Total creatinine generally manifested a moderate increase followed by a decline, but in no case did the elimination on the inadequate diet fall below the output on the adequate ration.

That the above effects are not to be attributed solely to the losses in weight of the rats on the deficient diets is indicated by the results of experiments in which a deficiency of tryptophane

was induced. When the ration was adequate in every respect except as regards its tryptophane content, the animals steadily declined in weight, but the output of allantoin and uric acid remained quite constant.

The addition of histidine to diets in which the component hydrolyzed casein had previously been subjected to silver precipitation led to increases in the excretion of total creatinine, uric acid, and allantoin, until the quantities eliminated were of the same order as in animals upon whole casein. On the contrary, the addition of arginine to the deficient rations failed entirely to affect the output of any of the urinary components. It is evident from these investigations, as well as from the growth experiments already described,⁴ that arginine and histidine are not interchangeable in metabolism. But as far as the relation of *histidine* to purine metabolism is concerned, our data confirm the findings of Ackroyd and Hopkins,⁵ and indicate that *under ordinary conditions of diet this amino acid is probably the mother substance of allantoin.*

Under the conditions which pertained in our experiments, *no relationship was observed between the arginine content of the of the diet and the total creatinine elimination in the urine.* As stated elsewhere,⁴ it is quite likely that the Kossel-Kutscher method of precipitation does not completely remove arginine. Assuming, therefore, that arginine is the precursor of creatine, it is possible that on the deficient diets our animals may have received adequate amounts of this amino acid for creatine anabolism, and that quantities fed in excess of the anabolic needs were catabolized by paths other than that which leads to creatine-creatinine production.

URIC ACID FORMATION IN THE CRUSTACEAN, PANULIRUS ARGUS.

By SERGIUS MORGULIS.

(From the Department of Biochemistry, University of Nebraska, Omaha.)

The blood of the crawfish *Panulirus* becomes entirely free of uric acid when it has been kept for a day or two without food. It has been found that if urea (1 to 3 gm.) is then injected intra-

⁴ Rose, W. C., and Cox, G. J., *J. Biol. Chem.*, 1924, lxi, 747.

⁵ Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, 1916, x, 551.

muscularly the uric acid reaction invariably reappears in the blood filtrate, the amount of uric acid frequently being in sufficient amount for quantitative determination. Administering the urea by way of the intestinal canal does not produce this effect. Likewise, experiments where various ammonium salts were used for the injection either failed to reproduce the results obtained with the urea or, as in the case of small doses of ammonium lactate, this result was obtained in a slight degree. Experiments performed with glycine, however, frequently did yield positive results, though in no instance were the effects as definite as in the case of urea injections. The blood uric acid was determined both by the Benedict direct method and by the method of Morris-Macleod, where the uric acid is precipitated preliminary to the development of the color. The results were essentially the same by both procedures. Space limitations do not permit a discussion of the reasons for the hypothesis that the uric acid is synthesized under the conditions of these experiments.

THE SYNTHESIS AND EXCRETION OF HIPPURIC ACID BY RABBITS.

By WENDELL H. GRIFFITH.

(From the Department of Biological Chemistry, St. Louis University, St. Louis.)

The rate of excretion of combined benzoic acid in the urine in the 6 hour period after the administration of sodium benzoate was determined for rabbits on a normal diet and on a diet to which thyroid powder was added. The addition of the thyroid powder (0.2 gm. of Armour's desiccated thyroids per day) to the diet resulted in an increased output of combined benzoic acid in the experimental period. This increased rate of excretion of combined benzoic acid might have been due to: (1) an increased rate of synthesis of glycine, or (2) to the presence of extra pre-formed glycine resulting from the breakdown of tissue protein, or (3) to an increased excretion of other forms of combined benzoic acid. A study of the nitrogen of the urine showed an increased total nitrogen and a marked creatinuria, but very little, if any, change in the hippuric acid nitrogen. The increased output

of combined benzoic acid was considered to be due to the presence of other forms of combined benzoic acid rather than to an increased synthesis and excretion of hippuric acid.

THE EFFECT OF REPEATED ADMINISTRATION OF SMALL AMOUNTS OF CYSTINE ON THE RABBIT.

By HOWARD B. LEWIS.

(From the Laboratory of Physiological Chemistry, University of Michigan, Ann Arbor.)

Cystine has been administered orally as the sodium salt in small doses (0.50 to 1.0 gm. per kilo) for successive days to fasting rabbits and to rabbits on a diet of oats and cabbage. After the 1st day protein and casts appeared in the urine, the excretion of total non-protein nitrogen and creatinine was depressed, but no marked increases in the amino acid and cystine excretion were observed. The blood showed a marked rise in the non-protein nitrogen and a slight increase in the amino nitrogen as determined by the Folin method.

THE OPTICAL ACTIVITY OF CYSTINE.

By JAMES C. ANDREWS.

(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)

The values previously recorded in the literature for the specific rotation of cystine in acid solution vary from -200° to -250° . It was found necessary to determine whether this variation was due to the conditions of the determinations or to the racemization which takes place under strongly acid conditions. In the present work variations in the kind of acid employed, its excess concentration, concentration of cystine, and concentration of other electrolytes present were studied. The results showed that proper variation of the above conditions can produce, in a single sample, specific rotation values ranging from -190° to -270° . Although for a given acid, the effect of change in pH and in cystine concentration was usually the same, several as yet unexplained anomalies were encountered and the effect of individual ions was found to be highly specific, particularly

when used in the form of salts. An extension of Patterson's theory of the effect of internal pressure on the form of asymmetric molecules in solution is advanced to account for the effects observed. In the present work the changes in internal solution pressure are produced by the varying degrees of hydration of the different ions present.

THE REACTION OF ACETOACETIC ACID WITH THE HEXOSES.

BY THEODORE E. FRIEDEMANN.

(From the Laboratory of Biological Chemistry, Washington University,
St. Louis.)

A further study was made of the ketolytic reaction described by Shaffer⁶ and by Shaffer and Friedemann,⁷ which is very similar to, and perhaps the basis for the phenomenon of antiketogenesis. Under optimum conditions in the presence of peroxide, alkali, and glucose, approximately 2 mols of acetoacetate are oxidized for each mol of glucose. Similar ratios are obtained with fructose, mannose, and glycol aldehyde.

In the presence of peroxide, *the rate of acetoacetate consumption and oxidation to acids, parallels the rate of oxidation of glucose.* This suggests that the active ketolytic derivative of the sugar is some oxidation product whose rate of condensation with acetoacetate is greater than its rate of further oxidation. However, one of the first oxidation products of glucose, glucosone, is not ketolytic in alkaline peroxide solutions, although it is ketolytically active in neutral or slightly alkaline solutions in the absence of H_2O_2 . In this respect it behaves similar to glyoxal, the oxidation product of glycol aldehyde.

This may indicate as interpreted by us in the case of the ketolytic reaction of glycol aldehyde that the condensation is not with an oxidation product but with a form of glucose, *before* oxidation. This is possible, since in high concentration glucose and acetoacetate do react, the rate paralleling the acid formation, most of which is CO_2 .

⁶ Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 433.

⁷ Shaffer, P. A., and Friedemann, T. E., *J. Biol. Chem.*, 1924, lxi, 585.

THE RATE OF ELIMINATION OF VARIOUS INGESTED MONO-SACCHARIDES IN PHLORHIZIN DIABETES.

BY H. J. DEUEL, JR., AND W. H. CHAMBERS.

(From the Department of Physiology, Cornell University Medical College, New York City.)

The hourly rate of elimination of "extra" glucose in the urine of dogs rendered diabetic with phlorhizin has been studied for 12 hour periods after the oral administration of glucose, galactose, fructose, and lactose in 16 gm. doses. The rates of excretion of glucose and galactose were practically identical with that of glucose itself, thus indicating that the intermediary transformations involved in the change of these monosaccharides to glucose is a very rapid one. The "extra" glucose recovered after glucose ingestion was 106 per cent of the theoretical in two experiments, 93 and 94 per cent after fructose and 88 per cent after galactose. With lactose, a 50 per cent recovery of glucose in 12 hours was obtained, but the hourly rate of excretion was slower and more constant.

A sparing action on the nitrogen metabolism was noted in each case despite the fact that none of the sugar was burned. The decreased nitrogen metabolism became evident the 2nd hour after the feeding of the carbohydrate and reached the minimum value 6 to 11 hours thereafter. The nitrogen level remained at this low value for several hours and then the amount eliminated gradually increased. It was noted that the mere ingestion of glucose by the fasting, phlorhizinized dog restored its muscular power even though the glucose was quantitatively excreted in the urine.

ON THE NATURE OF URINARY GLUCOSE.

BY W. C. AUSTIN AND T. E. BOYD.

(From the Laboratories of Physiological Chemistry and Physiology, Loyola University, Chicago.)

Female dogs were fasted and phlorhizinized. Fresh urine specimens were catheterized, clarified, and preserved. Comparisons of polariscopic measurements over long periods of time showed constant rotation, which coincided very closely with

copper reduction values for glucose content as estimated by the Shaffer-Hartmann method. Injections of insulin did not alter the ratios of polariscopic to copper reduction values. The conclusion is drawn that there is no mutarotatory ethylene oxide form of glucose in urine of phlorhizinized dogs, even under the influence of insulin. Is the nature of such urinary glucose the same as that of blood sugar?

ADEQUACY OF PIGEONS AND RATS FOR VITAMIN B STUDIES.

By A. D. EMMETT AND GAIL E. PEACOCK.

(*From the Medical Research Laboratories, Parke, Davis and Company, Detroit.*)

Data have been accumulated over a considerable period of time on the vitamin B requirements of pigeons and rats. Our experiments show that the pigeon, judged by the weight curve, as well as the prevention of polyneuritis, can be used to determine quantitatively the vitamin B, with as fair a degree of accuracy as the rat. Whether the basal diet of the pigeon is polished rice, or one high or low in carbohydrates, or the same as the regular vitamin B deficient ration generally given rats, does not seem to alter the conditions to any essential degree. When given a basal diet of polished rice supplemented with 0.00015 gm. of one of our concentrates, pigeons show a decided gain in weight, whereas, when the dose is reduced to 0.0001 gm., the birds are kept in equilibrium, maintaining their weight. The vitamin B maintenance requirements of mature pigeons always runs two to three times higher than the normal growth requirements of young rats.

QUANTITATIVE ASPECTS OF THE FUNCTION OF VITAMIN B IN SEVERAL SPECIES.

By GEORGE R. COWGILL, ARTHUR H. SMITH, AND H. H. BEARD.

(*From the Laboratory of Physiological Chemistry, Yale University, New Haven.*)

Data obtained from three widely different species of animals—mouse, rat, and dog—indicate that the vitamin B requirement is closely related to metabolism.

In the *adult* animal the vitamin B requirement is very closely proportional to the product of: (1) the two-thirds power of the weight—which may indicate surface or “active protoplasmic mass” or whatever the metabolism depends on, (2) the quantity of energy handled as indicated by the total calories utilized from the food, and (3) the body weight. The following formula has been constructed embracing these factors. By it a number called provisionally the “vitamin B constant” may be calculated.

$$\frac{\text{Vitamin B}_{\text{per day}}}{\text{Weight}^{\frac{2}{3}}} = K_{\text{vitamin}} \times \text{calories}_{\text{per day}} \times \text{weight} \quad (a)$$

whence

$$K_{\text{vitamin}} = \frac{\text{vitamin per day}}{\text{calories per day} \cdot \text{weight}^{\frac{2}{3}} \cdot \text{weight}} \quad (b)$$

$$K_{\text{vitamin}} = \frac{\text{vitamin}}{\text{calories} \cdot \text{weight}^{\frac{5}{3}}} \quad (c)$$

This formula gives good agreement with experimental data for *adult* rats, dogs, and mice. During *growth* some other factor, as yet undefined, appears to affect the vitamin requirement, K_{vitamin} during this period, having a higher value. Complete analysis of the growth phenomena may disclose this factor and enable a correction to be applied to this formula giving it more widespread significance.

The precise value of the vitamin “constant” just described is peculiar (1) to the species, the value being greatest with the mouse, and smallest with the dog, (2) to the source of vitamin B used in the test, and (3) to the particular set of conditions maintained in the investigation. Under any given set of experimental conditions the value is constant within the limits of error of the accepted methods of vitamin testing.

In view of the wide variation in growth rate and food intake shown by rats and mice, particularly when the vitamin B supply is close to the minimum, statistical treatment of the data becomes necessary. This requires that voluminous data be secured in studies of this nature.

ANTIRACHITIC ACTIVATION BY LIGHT.

By H. STEENBOCK, A. BLACK, E. M. NELSON, M. T. NELSON,
AND C. A. HOPPERT.

*(From the Department of Agricultural Chemistry, University of Wisconsin,
Madison.)*

Using two methods of testing for the presence of antirachitic activation; namely, (a) the induction of growth and calcification in the rat during phosphate inhibition and (b) the cure of rickets produced by deficient phosphate and excess of calcium in the diet, it has been found that a large number of naturally occurring materials can be activated antirachitically by exposure to ultra-violet light. Among these are yeast, commercial casein, ox bile, lanolin, and grains and their commercial products such as starch, meals, flours, breakfast foods, and oils.

The antirachitic activation apparently cannot be induced in pure protein, carbohydrate, or fat or such substances as salts, water, ether, hydrochinon, phloroglucin, or paraffin hydrocarbons. It appears to be localized in the unsaponifiable lipoidal constituents as the sterols and closely related compounds. Cholesterol purified first by numerous crystallizations and then as a benzoate and finally as an acetate can be activated. Phytosterols cannot always be activated. Digitonin will not precipitate all the active constituents from the unsaponifiable fraction of cod liver oil.

It follows: (a) that a definite function for cholesterol has been discovered; and (b) that irradiation of naturally occurring or synthetic compounds is available as a new principle in the pharmacotherapy of diseases known to respond to direct irradiation.

**A FURTHER REPORT ON IMPARTING ANTIRACHITIC PROPERTIES
TO INACTIVE SUBSTANCES BY ULTRA-VIOLET IRRADIATION.**

By ALFRED F. HESS AND MILDRED WEINSTOCK.

*(From the Department of Pathology, College of Physicians and Surgeons,
Columbia University, New York.)*

Activity has been induced in linseed or cottonseed oil by irradiation with the mercury vapor lamp and was found to be practically unimpaired in tests carried out 6 months later. The

oil likewise acquired antirachitic properties when irradiated in an atmosphere of nitrogen. As in cod liver oil the specific principle of the activated oil was found to be present in the non-saponifiable fraction; it was absent in the non-saponifiable fraction of ordinary non-irradiated oil. Glycerol, gelatin, and milk (in the small doses fed) were not rendered antirachitic by irradiation. Wheat, whether green or etiolated, was endowed with the antirachitic property by means of irradiation and maintained this property for a period of at least 2 weeks. The same is true in regard to green or yellow lettuce which, under ordinary conditions, has no protective value. Potency is retained in these vegetables at least for several days. Refined wheat flour can be rendered active by this means. Cholesterol, as well as phyto-sterol, was found to have no antirachitic virtue when fed to rats, but after they had been exposed to irradiation, parallel tests showed that they had acquired this property.

DIETARY REQUIREMENTS FOR REPRODUCTION.

IV. POSITIVE EVIDENCE FOR THE EXISTENCE OF A REPRODUCTIVE DIETARY COMPLEX (VITAMIN E) SOLUBLE IN ETHER, BENZENE, AND ACETONE.

By BARNETT SURE.

(From the Department of Agricultural Chemistry, Agricultural Experiment Station, University of Arkansas, Fayetteville.)

No fertility was secured with the control ration having the following composition.

	<i>per cent</i>
Skimmed milk powder.....	50.0
Ferric citrate.....	0.2
Agar-agar.....	2.0
Harris yeast-vitamin powder (later increased to 1.0 per cent) ..	0.5
Cod liver oil.....	2.0
Dextrin.....	45.3

When 5 per cent of dextrin was replaced by 5 per cent of either ether, acetone, or benzene extracts of the wheat embryo fertility was always obtained, but, in order to secure absolute success in lactation, the Harris yeast-vitamin powder, which was used as a source of water-soluble B vitamin, had to be increased to 1.0

per cent of the total ration. Healthy second generations are growing up normally on skimmed milk powder rations containing 3 and 5 per cent of the wheat germ oil.

Wheat embryo extracted with ether, acetone, or benzene produces fertility (on account of incomplete extraction of all the fat as shown by chemical analysis), but is inadequate for lactation. The addition of acetone or ether extracts of the wheat embryo to wheat germ preparations extracted with such organic solvents results in success not only in fertility but also in rearing of young.

The evidence presented clearly shows the existence of a specific dietary complex essential for reproduction which is soluble in organic solvents, and it is proposed that this vitamin be termed "fat-soluble E."

THE INFLUENCE OF MILK RATIONS HIGH AND LOW IN FATS ON THE SEX GLANDS OF MALE ALBINO RATS, WITH SPECIAL REFERENCE TO SUBSTANCE X.

BY H. A. MATTILL AND M. M. CLAYTON.

(From the Department of Vital Economics, The University of Rochester, Rochester, New York.)

The prevention of sterility in male rats on milk rations by substance X or by lowering the fat content of the ration is confirmed. Table I summarizes the data on gonad weights secured from some of the male animals examined after 140 days of age.

TABLE I.

	Total No. of animals.	Those having normal gonads.	Those having gonads less than 80 per cent of normal weight.
High fat ration	41	7	34 (83 per cent)
" " " plus X* 1st generation	10	7	3 (30 " ")
" " " " " 2nd " "	15	14	1 (6 " ")
Low fat ration	16	10	6 (38 " ")
" " " 2nd generation.....	5	0	5 (100 " ")
Stock colony animals.....	38	35	3 (8 " ")

* Unextracted wheat embryo or lettuce leaves.

These figures also indicate that on rations low in fat, X cannot be dispensed with. It must be supplied in high fat rations during adolescence and early maturity. Of fifteen animals transferred from inadequate to adequate diet after 130 days of age, only one failed to show degeneration. Adequate food for as long as 200 days thereafter failed to restore fertility. Conversely, of ten animals transferred from adequate to inadequate rations before 90 days of age, only one escaped degeneration. Of fourteen so transferred after 90 days of age only three suffered degeneration. Substance X therefore appears to be required for the establishment of normal structure and function of the testes, but thereafter it is less critically needed. Of the unsaponifiable constituents of wheat embryo oil those transformable into cholesterol by the animal probably do not include X for the blood cholesterol of animals showing degeneration tends to be higher than that of normal animals.

MINERAL METABOLISM OF ADULT MAN.

BY GUY W. CLARK.

(From the Departments of Biochemistry and Pharmacology, University of California, Berkeley.)

The work reported in this paper represents only one phase of a group attack^a being made to determine, if possible, the direct causative and contributory factors involved in pyorrhea alveolaris.

Since the literature does not reveal any detailed and complete experiments in the field of mineral metabolism it becomes necessary to study such changes in normal individuals before any attempt is made to study similar processes where pathological changes are in progress.

Four adult males, representing different ages and of different temperaments, served as experimental subjects. The experiments were carried on for 28 weeks, during which time five different diets were fed. Composite samples of each of the foods were collected. A weekly urine composite was obtained by taking one-tenth of

^a This work, made possible by a grant from the Carnegie Corporation to the University of California, is being carried on by the California Stomatological Research Group.

each day's excretion. Feces were dried and made into weekly composites. From the analyses of these composite samples weekly balances are being prepared for the following elements: calcium, magnesium, sodium, potassium, chlorine, phosphorus, sulfur, and nitrogen.

Blood analyses, which included calcium, magnesium, sodium, potassium, chlorine, acid-soluble phosphorus, and carbon dioxide, were made twelve times during the 28 weeks.

Not knowing how changes in the character of a diet might affect the chemical composition of the saliva, weekly samples of "resting" saliva were analyzed for the following elements: calcium, magnesium, sodium, potassium, chlorine, sulfur, and acid-soluble phosphorus. Determinations of total solids, organic matter, ash, and carbon dioxide were also made.

A complete report of this work will be made in the near future.

CALCIUM AND PHOSPHORUS METABOLISM IN DAIRY COWS.

BY EDWARD B. MEIGS AND WILLIAM A. TURNER.

(From the Dairy Division Experiment Station, United States Department of Agriculture, Beltsville.)

The calcium, phosphorus, and nitrogen balances have been followed continuously in two milking cows for a period of 175 days. During the whole of this period the animals were fed liberal amounts of grain, to which were added various kinds of hay. During 3 weeks of the experiment the rations were supplemented with cod liver oil.

The kinds of hay used were timothy and two grades of alfalfa. One lot of the alfalfa used had been well cured with no exposure to rain and with only a short exposure to direct sunlight. The other lot had been allowed to lie in the field for a week, and had been rained on for nearly 2 days before being dried. The good alfalfa contained about 2.3 per cent calcium; the poor alfalfa about 2.0 per cent; the timothy, about 0.3 per cent; and the grain, about 0.15 per cent.

While fed the good alfalfa, the cows remained fairly close to calcium equilibrium. On the poor alfalfa and timothy, they lost calcium rapidly from their bodies. The losses on the poor alfalfa were somewhat more rapid than those on the timothy hay

in spite of the much higher calcium content of the former. The phosphorus metabolism followed the calcium in a general way, but by no means exactly.

In the course of 133 days on poor alfalfa and timothy hay, the cow giving the larger amount of milk lost about 1,300 gm. of calcium from her body, or about 19 per cent of the total normal calcium content of a cow's body. She was still giving 10 kilos of milk daily at the end of this period, and was losing calcium nearly as fast as she had at the beginning.

100 cc. of cod liver oil added daily during 21 days to the ration of grain and poor alfalfa hay did not improve the calcium assimilation in either cow, though it produced a noticeable increase in milk yield in one of them.

THE CALCIUM CONTENT OF THE BODY IN RELATION TO AGE, GROWTH, AND FOOD.

By H. C. SHERMAN AND F. L. MACLEOD.

(From the Department of Chemistry, Columbia University, New York.)

The normal calcium content of the albino rat has been established by the analysis of large numbers of animals at definite ages from birth until full maturity. The percentage of calcium in the body averages about 0.25 per cent at birth and increases to from 1.0 to 1.2 per cent in the normal adult. Females which had not raised young contained a slightly higher average percentage of calcium than males of the same ages which had lived on the same diet and under the same conditions. The bearing and suckling of young diminished the calcium content of the mother's body. The effects of several differences in diet upon the calcium content of the body are described and discussed.

AGE CHANGES IN THE CHEMICAL COMPOSITION OF THE LONG BONES.

By FREDERICK S. HAMMETT.

(From The Wistar Institute of Anatomy and Biology, Philadelphia.)

In order to obtain an insight into the chemical processes concerned in bone formation during growth, and in order to provide standard data with which experimental alterations of bone com-

position, such as occur in rickets, can be compared, a determination was made of the water, organic matter, and ash; and the calcium, magnesium, and phosphorus content of the ash of the humerus and femur of normal male and female albino rats at the ages of 23, 30, 50, 65, 75, 100, and 150 days. The bones of ten rats of each sex of each age were analyzed.

It was found that the organic matter and ash percentage increase steadily with age, while the water percentage decreases. The curves of the ash increment and the water decrement are similar to that of an autocatalyzed monomolecular reaction. A slight disturbance in the course of ossification occurs in the females during puberty, which is not detectable in the males.

The percentage of ash and organic matter is greater in the bones of the females than in the males through the growth period observed. The water percentage is less in the females than in the males.

The composition of the ash changes with age. The percentage of calcium increases. The percentage of magnesium and phosphorus tends to decrease. This holds for both bones in both sexes. A disturbance of calcification is suggested at puberty in both sexes. No consistent sex difference in percentage of magnesium or phosphorus is detectable during the growth period observed. The calcium percentage tends to be greater in both bones of the females, save at 30 and at 75 days of age. These are critical times in the physiological development of the albino rat as has already been shown from the refractometric studies of blood serum by Hatai.

STUDIES ON CHOLESTEROL.

II. INFLUENCE OF CHOLESTEROL ON NUTRITION AND GROWTH.

BY ARTHUR KNUDSON AND F. S. RANGLES.

(From the Laboratory of Biological Chemistry, Union University Medical Department, Albany Medical College, Albany.)

In a previous study we have demonstrated that cholesterol is synthesized in rats on a cholesterol-free diet. White rats were placed at the time of weaning on a cholesterol-free diet, but containing protein, carbohydrate, traces of fat, inorganic

salts, and sources of vitamins A and B. On these diets good growth was obtained indicating that cholesterol is not essential for growth. To the cholesterol-free diets known amounts of cholesterol were added and there was no appreciable difference in their nutrition as evidenced by their rate of growth. The extracted cholesterol-free diet was deficient in the so called vitamin X of Evans, as evidenced by the fact that reproduction was very poor on this diet and that the number of litters born was considerably less than normal. Addition of cholesterol to this diet improves the fertility as shown by the fact that the number of litters born was greater and also that we have so far obtained a third generation while the second generation of rats on the cholesterol-free diet have failed to reproduce.

THE EFFECT OF WAKING ON URINARY CHLORIDES AND pH;
SHORT INTERVAL URINES DURING THE SECOND DAY
OF FASTING.

By GEORGE ERIC SIMPSON.

*(From the Department of Biochemistry, McGill University, Montreal, and
the Department of Physiological Chemistry, University of
Pennsylvania, Philadelphia.)*

If an experiment be so arranged that urine volume shows no marked change on waking in the morning after a night's sleep, it can be shown that hourly chloride excretion and urine pH definitely increase. When a subject stays awake all night and sleeps from 7 to 11 in the morning, urinary chlorides and pH decrease when the subject falls asleep, if the subject sleeps well at this abnormal hour. More often sleep is fitfull, and urinary chlorides and pH remain practically unchanged. Experiments are under way which it is hoped may determine if these results are to be explained on the basis of a salt shift from tissues or corpuscles to serum, and which is simultaneous with the demonstrated blowing off of CO₂ on waking.

Earlier work on short interval urines has been extended to include the 2nd day of fasting. The urine volume curve does not show the sharp variations exhibited during the 1st day of fasting. It is relatively smooth. Maxima for chlorides, phosphates, titratable acidity, and organic acids in the afternoon

are about double the minimum level for these substances, found during the night. The curves show the same general contour, and resemble curves previously determined for phosphates by Fiske and by Broadhurst and Leathes. Such variations cannot be regarded as peculiar to phosphates.

CYCLIC VARIATIONS IN THE COMPOSITION OF FASTING BLOODS IN WOMEN.

BY RUTH OKEY.

WITH THE COOPERATION OF STATIE E. ERIKSON, RUTH E. BOYDEN, ELDA
I. ROBB, AND THELMA PORTER LEVIN.

*(From the Laboratory of Household Science, University of California,
Berkeley.)*

The blood analyses here reported have been a part of a more general study of the variations in the metabolism of women in relation to the menstrual cycle. A group of twenty-five women students, normal in as far as could be determined by inspection of the records of the routine university physical examinations, have served as subjects. Three of these were on weighed and analyzed diets, constant for from 1 to 3 months at a time, for totals of 3, 5, and 6 months, respectively. The others were instructed to make as little variation as possible in their ordinary routines of diet and exercise while under observation. Blood samples were always taken before breakfast in the morning, and under as nearly uniform conditions as possible. The ordinary technique of the Folin-Wu system of analysis was followed unless otherwise noted.

Blood uric acid, as far as can be judged from a total of 280 determinations by the Benedict direct method, extending through forty monthly cycles, rises, just before or at the time of the onset of menstruation, to a level which averages 6 per cent above that for the intermenstrual period. This is followed, within from 1 to 3 days, by a fall to approximately 86 per cent of the average intermenstrual level for the individual. Within from 3 to 7 days after this fall, the uric acid rises to a level which is usually almost as high as that reached before menstruation. Following this latter rise, a decline to approximately the average intermenstrual level usually takes place within a few days. Ex-

treme high and low values observed were 127 and 57 per cent, respectively, of the intermenstrual levels for the individuals concerned.

In approximately half of these blood filtrates, *uric acid* was also determined by the Morris-Macleod method. The curves so obtained showed the same type of premenstrual rise, menstrual fall, and postmenstrual rise, but they were not, in some cases, parallel to those based on the analyses by the other method. Data do not, however, justify at this time a discussion of the extent to which the occurrence of different forms of uric acid combinations in the blood may be responsible for these variations.

On a mixed diet which included purines, the menstrual variation in blood uric acid tended to be greater than that of the same individual on a purine-free diet. Moreover, while on long continued purine-free dietary regimes the pre- and postmenstrual rises in uric acid were still marked, the menstrual falls did not usually reach levels much below those of the average intermenstrual values. The number of cases on controlled diets is, however, too small to warrant any definite conclusions based on this observation. It is, moreover, unfair to assume that blood samples taken even as often as once a day will show the maximum effect of menstrual variations in any individual all the time.

Non-protein nitrogen values usually rose and then fell during menstruation, but these curves were neither so striking nor so uniform as those from the uric acid determinations. Only a part of the samples have been analyzed for *urea*, but these showed no evidence of a significant menstrual variation. Analyses of blood *creatine* and *creatinine* have, in general, confirmed the observations of Wang and Dentler⁹ to the effect that there is no significant relationship between variations in the levels of these substances and the menstrual period.

The average values for fasting *blood sugars* tend to be slightly higher (1 to 8 mg. per 100 cc.) just before or during menstruation than at any other time. There is also a tendency, however, to larger deviations from the average values for blood sugar during this period. Hence, although a total of approximately 300 determinations has been made, any conclusion that there is a uniform variation in blood sugar level in relation to the men-

⁹ Wang, C. C., and Dentler, M. L., *J. Biol. Chem.*, 1920-21, xlv, 237.

strual cycle seems hardly justified by the data obtained. That the time of the menstrual period should, in as far as possible, be avoided in making blood sugar determinations for purposes of clinical diagnosis, does, however, seem to be indicated.

A study of monthly variations in blood lipoids as determined by Bloor's revised methods of analysis has included approximately twenty-five cycles in fifteen subjects. A tendency to a rather abrupt fall in blood *cholesterol* (amounting, usually, to from 40 to over 100 mg. per 100 cc. of blood) during a period covering from 3 to 10 days before the beginning of the menstrual flow is indicated. After the onset of menstruation, the blood cholesterol begins to rise, usually somewhat abruptly at first then more slowly. Although there are day to day fluctuations of smaller magnitude, the general trend of the cholesterol curve usually continues to be upward until the period just preceding the next menstruation, when a fall again occurs. It is suggested that the time of this fall in blood cholesterol may be coincident with that of the most rapid growth of the corpus luteum, while the rise in the cholesterol level marks the period of its retrogression.

A rise in *fatty acid* was sometimes observed during or just preceding the fall in cholesterol, but this was by no means consistent for all cases. *Blood lecithin* showed little or no variation in relation to the menstrual period. Unfortunately, the amounts of the samples available have been too small to permit of separate analyses of plasma and corpuscles. A study of free cholesterol and cholesterol ester ratios is in progress.

**PRELIMINARY REPORT ON THE BASAL METABOLISM OF 157
NORMAL SCHOOL CHILDREN BETWEEN THE AGES OF FIVE
AND SEVENTEEN YEARS.**

By IRENE SANDIFORD AND ETHEL R. HARRINGTON.

(From the Section on Clinical Metabolism and the Section on Pediatrics,
Mayo Clinic, Rochester, Minnesota.)

In order to establish with greater accuracy the standard basal metabolism of children we are carrying out metabolism studies on the school children of Rochester, Minnesota; thus far we have examined 79 girls (231 determinations) and 78 boys (224 deter-

minations) quite evenly distributed between the ages of 5 and 17. The children were carefully selected by one of us (E. R. H.) and were in good health, although the routine determination of the oral temperature revealed a slight elevation of 1° in a small percentage of the tests. The children were very good and cooperated in practically all instances. They came to the laboratory at 7.15 a.m., without breakfast, and were allowed to rest half an hour before the metabolism determination was made. The gasometer method with analysis of the expired air was used according to the technique fully described elsewhere by Boothby and Sandiford. Single determinations were made on 3 successive days, except in a few cases in which the children came only once or twice.

The data, which have been plotted in various ways, will be presented in detail in the final paper. The results are expressed as calories for each square meter, using the Du Bois height-weight formula. As a rule the higher determination was obtained on the 1st day and generally the two subsequent determinations agreed more closely. A few of the higher determinations will be eliminated in drawing the average because of a slight elevation of the temperature or restlessness at the time of the determination. For comparison with the results of the other workers their averages are indicated on the chart by the various lines. As the study is still in progress we have thought it inadvisable at the present time to draw our own averages. However, it is obvious that our results for both boys and girls are falling between the upper triple line representing the Du Bois standards, and the lower triple line which indicates the Benedict-Talbot data. If only the lowest value of each one of the children is considered the general average for each year will be closer to the Benedict-Talbot than to the Du Bois line. However, the validity of omitting all but the lowest determination is open to question, when attempting to determine the best standard for clinical work. Besdale's results are, on the whole, lower than ours for both boys and girls, and this is also true of MacLeod's findings on girls of from 11 to 14. It is interesting to note that there is slightly less scattering of our data (not only of the single tests but also of the individual) among the girls than among the boys, probably owing to the fact that boys are more restless and

more interested in watching the technical procedures than girls. The decrease in calories for each square meter progresses very regularly, and appears to be nearly, if not absolutely, a straight line function of age for both boys and girls from the ages of 5 to 17, and in this respect agrees more closely with the findings of Benedict and Talbot than with those of Du Bois.

THE INHIBITION OF AUTOLYSIS BY PROTEIN.

By A. B. HERTZMAN AND H. C. BRADLEY.

(From the Department of Physiological Chemistry, University of Wisconsin, Madison.)

The addition of certain foreign proteins to an autolyzing liver brei inhibits the digestion of the liver, and the foreign protein itself is not digested. Egg albumin or the serum proteins give pronounced inhibitions of this sort. The inhibition is roughly proportional to the mass of foreign protein added, though never complete within the limits of our experiments. Other native proteins, such as edestin, produce no inhibition, and are themselves hydrolyzed.

The factor determining whether a foreign protein inhibits autolysis or not, is apparently the isoelectric point of the protein and its relation to the pH of the autolyzing liver. If the reaction of the liver brei approaches the pH value of the isoelectric point of the added protein, it does not inhibit and is itself digested. If, however, the isoelectric point of the foreign protein is more acid than the reaction of the brei, the protein inhibits autolysis.

We believe the explanation is that the protease distributes itself between the proteins present in the mixture and that the fraction fixed by the base-protein is in relatively stable combination. No hydrolysis results and no dissociation of the protease. The mixture thus has less active catalyst present than where no foreign protein was present. The autolytic decomposition of the liver, therefore, goes on more slowly than in the control, because there is less enzyme.

This, we believe, is additional evidence that in the normal living cell with its normal pH about 7 there is no active primary protease. It is present in stable base-protein-enzyme combina-

tion and can only be liberated by a rise of the H ion concentration.

The instability of the acid-protein-enzyme complex seems to be associated with a weakening of the peptide linkage such as might be assumed with a change in the valence of nitrogen in the imino group, from 3 to 5 with the addition of acid. The protein breaks hydrolytically at this point and the enzyme is also set free.

A METHOD OF OBTAINING FROM VEINS BLOOD SIMILAR TO ARTERIAL BLOOD IN GASEOUS CONTENT.

By SAMUEL GOLDSCHMIDT AND ARTHUR B. LIGHT.

(From the Department of Physiology, University of Pennsylvania, Philadelphia.)

We have shown that blood taken from veins on the dorsal surface of the hand more nearly approximates arterial blood in its oxygen saturation and carbon dioxide content than does blood taken from veins in the antecubital space. In addition, when the hand and wrist are immersed in water at 45–47°C. and allowed to remain for 10 to 15 minutes, the oxygen saturation of the venous blood from the part is markedly increased.

Our results show that, because of the normally low oxygen unsaturation of blood from veins on the back of the hand, the application of heat, by increasing the blood flow through the hand, causes the arterial blood to pass into the veins with an indistinguishable change in its gaseous content beyond the limits of error of the methods of determination used.

Comparisons of blood, obtained under these conditions, from a vein on the dorsal surface of the hand, with blood from the brachial or radial artery have been made in six individuals. In nine other experiments, where only the venous blood was drawn, from veins on the back of the hand after exposure to hot water, figures of oxygen saturation within the range for arterial blood were obtained.

**ON THE DETERMINATION OF NON-PROTEIN NITROGEN IN
VERY SMALL AMOUNTS OF BLOOD.**

BY JOSEPH C. BOCK AND MAX GILBERT.

*(From the Department of Physiological Chemistry, Marquette University,
Milwaukee.)*

Several drops of blood (80 to 150 mg.) are obtained from the tip of a finger or the lobe of the ear. The blood is collected in a small, thin glass tube and weighed. We prefer the use of a torsion balance, because the weighing can be done in such a short time that no anticoagulant is necessary:

The blood sample is transferred to a 4 ml. centrifuge tube and proteins are precipitated with a mixture of trichloroacetic acid and kaolin. After centrifugation and filtration an aliquot part of the filtrate is heated in a test-tube with a sulfuric acid digestion mixture. The ammonia is liberated by the addition of alkali, distilled by a micro steam distillation, and determined by Nesslerization.

**THE DETERMINATION OF ARGININE BY THE USE OF ARGINASE,
WITH APPLICATIONS TO THE ANALYSIS OF PROTEINS AND
THE STUDY OF TRYPTIC DIGESTION.**

BY ANDREW HUNTER AND JAMES A. DAUPHINEE.

*(From the Department of Biochemistry, University of Toronto, Toronto,
Canada.)*

Under suitable conditions arginase effects a practically complete conversion of arginine into ornithine and urea. It is therefore possible to determine arginine by the successive use of arginase and urease. A method based upon this principle has been applied (a) to the study of the rate at which arginine (or an arginine peptide susceptible to the action of arginase) is liberated during the course of tryptic digestion, and (b) to the determination of the arginine content of proteins.

(a) When 1 gm. of active "trypsin" is added to 250 cc. of a 5.5 per cent solution of *gelatin*, one-third of the total arginine is split off within the first 30 minutes; in 3 hours more than half has been liberated; and by the 3rd day an equilibrium has been reached, in which almost exactly two-thirds of the total arginine

is free. A similar astonishingly rapid liberation of arginine has been observed in the digestion of *casein*; and here also just one-third of the arginine linkages appear to be resistant to the action of trypsin. With *edestin* the appearance of arginine is much more gradual; the process has not yet come to an end on the 6th day, at which point of time only 50 per cent is free.

(b) The arginase method may be applied to the solution of bases obtained in the Van Slyke method of protein analysis, where it possesses certain advantages over the usual procedure for the determination of arginine. As an example of the results obtained by its use, we have found for casein 7.7, 7.9, 8.0, 7.9, and 7.9 per cent of the total nitrogen in the form of arginine. The figure reported by Van Slyke (corrected, like our own, for the solubility of arginine phosphotungstate) is 8.1.

THE OCCURRENCE OF AMINO ACIDS AND OTHER ORGANIC NITROGEN COMPOUNDS IN LAKE WATERS.

By W. H. PETERSON, E. B. FRED, AND B. P. DOMOGALLA.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

Large quantities of water from Lake Michigan and four inland lakes of Wisconsin were centrifuged to remove plankton and bacteria, concentrated in a vacuum to 1/200 of the original volume, and analyzed for various forms of organic nitrogen.

Good color tests for proteins were obtained with ten different reagents. Proteins and their decomposition products were also shown to be present by precipitation with a number of protein precipitants. The existence of free amino nitrogen and peptide nitrogen was established by means of the Van Slyke, Folin, and Sørensen methods.

The quantity of certain amino acids was determined after hydrolysis of the concentrated sample and was found to range per cubic meter of water as follows: tryptophane from 5.5 mg. in Lake Michigan to 16.4 mg. in Green Lake; tyrosine from 8.3 mg. in Lake Michigan to 17.6 mg. in Devil's Lake; histidine from 5.7 mg. in Lake Mendota to 22.7 mg. in Turtle Lake; cystine from 1.5 mg. in Lake Mendota to 7.5 mg. in Turtle Lake.

Small quantities of amines and purines were also found. From 90 to 95 per cent of the organic nitrogen was accounted for by the different forms determined.

**SOME OBSERVATIONS ON THE INTERRELATION BETWEEN THE
FUNCTIONAL LEVELS OF THE ANIMAL BODY AND THE
EXTERNAL COOLING POWER.**

By E. S. SUNDSTROEM.

(From the Department of Biochemistry, University of California, Berkeley.)

Genetically related rats, which in every other respect were treated similarly, were given ample time to adapt themselves to a series of artificially produced climatic environments, which were maintained so as to give a gradient of cooling power; namely, (1) ordinary room temperature and humidity with air in motion (dry Kata 7, wet Kata 19), (2) high temperature and high humidity plus draft from electric fans (dry Kata 2, wet Kata 9), high temperature and low humidity (dry Kata 0.9, wet Kata 7), high temperature and low humidity, no "breeze" (dry Kata 0.8, wet Kata 4).

With the progressive drop in cooling power rather definite gradients were observed in the numerical data dealing with a number of morphological characters and functional levels. *Decreasing* proportionately with a lowered cooling power were: body weight, relative weight of internal organ, for instance liver, kidneys, spleen, and *thyroid*, resting respiratory metabolism, food consumption, blood sugar, blood uric acid, total creatinine of blood, nucleotide nitrogen of the blood, acid-soluble and lipoid phosphorus, and lecithin:cholesterol ratio. *Increasing* with the falling cooling power were: blood urea and to some extent amino acids, chlorides, partial blood cholesterol and relative weight of adrenals, body temperature of females (males preserved the body temperature better), and also a number of body indices, which appear to be positively correlated with the skin area. Besides, the coat of the white rats showed in the hot environments a reddish creamy coloration, the depth of which tended to increase along the cooling power gradient.

INORGANIC PHOSPHORUS IN INFANT BLOOD.

By ANTON R. ROSE, EDWIN A. RIESENFELD, AND I. HANDLEMAN.

(From the Prudential Life Insurance Company, Newark.)

The arterial chord blood is consistently richer in inorganic phosphorus than venous chord blood. The average difference is only 4 per cent. The average of the inorganic phosphorus of the arterial and venous chord blood is in very close agreement with that of the mixed chord blood as usually taken (average difference 0.06 mg.). Blood samples from different anatomic levels will probably give different values. In a few cases the differences will be less than 10 mg. Between chord and fontanel the chances of one exceeding the other in inorganic phosphorus are about equal, but when the phosphorus of the fontanel is the larger in amount the differences are more pronounced, ranging from 10 to 75 per cent as against 10 to 30 per cent. The mass of blood cells runs parallel to the inorganic phosphorus so that if the percentage of blood cells be divided by milligrams of inorganic phosphorus per 100 cc. a constant number will be got which is nearly the same for all the samples of a given case, but not necessarily the same for different infants. The average of this constant for all infants comes to 13. The individuals range from 11.5 to 15.

A STUDY OF THE BIREFRINGENCE AND THE STAINING OF AGAR-AGAR AND OF GELATIN.

By JOHN FIELD, 2ND, AND C. L. ALSBERG.

*(From the Food Research Institute and the Department of Chemistry,
Stanford University, California.)*

The writers have shown recently that agar-agar dried in the gel form is very strongly birefringent, whereas dried in the sol form it is not birefringent at all except somewhat at the periphery or at the edges of a crack. It can now be reported that gelatin behaves in exactly the same way and that the phenomenon is not due to the production of strain by adhesion to the support during drying and shrinking. Preparations dried on oiled glass or on paraffin blocks do not adhere to them, yet show the properties above described. It is suggested that the birefringence of dried gels may need to be taken into consideration in interpreting some

of the recorded observations upon the optical properties of dried gelatin.

The dried agar gels were quickly and deeply stained by alcoholic solutions of many of the dyes used in cytological studies, whereas the dried sols stained but slowly if at all. It is suggested that this phenomenon may be significant in cytological work. Furthermore, a hypothesis in explanation of the contrasting behavior of dried gels and sols, as recorded in the present investigation, is offered.

THE PURIFICATION OF JACK BEAN UREASE.

By J. B. SUMNER AND V. A. GRAHAM.

(From the Departments of Physiology and Biochemistry, Cornell University Medical College, Ithaca.)

The authors have continued the work of Sumner, Graham, and Noback.¹⁰ One part by weight of jack bean meal is extracted at 20°C. with 2 volumes of 30 per cent alcohol. The press-juice is made 35 per cent alcoholic and centrifuged. The centrifuged extract is allowed to stand in a tall cylinder overnight at -10°C. The supernatant liquid is syphoned off and discarded and the precipitate is centrifuged while cold. The precipitate, containing nearly all of the urease, is stirred up with 30 per cent alcoholic phosphate solution of the same pH as the original extract. The material is cooled at -10°C. for $\frac{1}{2}$ hour and centrifuged. This process is repeated five times more and removes all carbohydrate and some protein. The precipitate is now stirred up with a small amount of dilute aqueous neutral phosphate solution and seeded with crystals of concanavalin A. After 48 hours at 3-5°C. all of the concanavalin A and concanavalin B will have crystallized out. These crystalline proteins are centrifuged off. The product has been dialyzed to remove phosphates, dehydrated with alcohol and ether, dried, and analyzed for total nitrogen and ash and for cystine, tyrosine, and tryptophane by the methods of Folin and Looney.¹¹ The results indicate that urease is of protein nature and that it is not identical with canavalin, concanavalin A, or concanavalin B. Urease can be purified

¹⁰ Sumner, J. B., Graham, V. A., and Noback, C. V., *Proc. Soc. Exp. Biol. and Med.*, 1923-24, xxi, 551.

¹¹ Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.

further by conversion to insoluble urease by the action of dilute alcohol. The authors are at present engaged in analyzing insoluble urease.

**PROTEINS OF THE BARK OF THE COMMON LOCUST TREE,
ROBINIA PSEUDACACIA, LINN.**

By D. BREESE JONES AND C. E. F. GERSDORFF.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

The first recorded instance in which protein has been shown to occur in the bark of a tree is that noted by Power and Cambier,¹² who isolated from the bark of the common locust a protein having toxic properties. This protein was subsequently designated by the name *robin*. Numerous instances are recorded of both people and domestic animals having suffered as a result of chewing or eating the bark of this tree.

In view of the twofold interest connected with the protein of the locust bark, namely its toxic properties, and the fact that it opens a new and a wide field for protein investigation in a domain to which but little attention has been hitherto paid, we have undertaken a study of its proteins and other nitrogenous constituents.

The bark was stripped from the trees in August, and after carefully removing the outer corky portion, it was allowed to dry in the air, and finally ground to a coarse meal. The ground bark contained 2.8 per cent of nitrogen (moisture-free).

The results of exhaustive extractions of the bark made at room temperature are given in Table I.

TABLE I.

Percentages based on the moisture-free bark used.

Solvent.	Per cent of total N.	Proteins as per cent of the bark ($N \times 6.25$).
Distilled H ₂ O.....	54.69	9.57
10 per cent aqueous NaCl.....	9.36	1.64
70 " " alcohol.....	0.67	0.12
0.5 " " aqueous NaOH.....	1.34	0.23
Total.....	66.06	11.56

¹² Power, F. B., and Cambier, J., *Pharm. Rundschau*, 1890, viii, 29.

There have been isolated from the bark 2.52 per cent of albumin, 1.38 per cent of a globulin, and a quantity of a substance having the properties of a proteose.

The albumin is completely precipitated from a 10 per cent salt solution by making the solution 0.4 to 0.5 saturated with ammonium sulfate. On heating its salt solution the albumin coagulates at 61° to 62°C. The average results of elementary analyses of nine different preparations showed the albumin to have the following composition: C 54.52, H 6.83, N 14.76, S 0.80.

The distribution of nitrogen and percentages of diamino acids in the albumin as calculated from results obtained by the Van Slyke method of analysis are expressed in Table II as percentages of the protein. Cystine, tryptophane, and tyrosine were determined colorimetrically.

TABLE II.
Locust Bark Albumin.

	<i>per cent</i>
Amide N.....	1.41
Humin N.....	0.53
Basic N.....	3.09
Non-basic N.....	9.95
Cystine.....	1.37
“.....	1.03*
Arginine.....	4.39
Histidine.....	1.74
Lysine.....	5.45
Tryptophane.....	4.18†
Tyrosine.....	6.27*

* Determined by the colorimetric method of Folin and Looney (Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421).

† Determined by the colorimetric method of May and Rose (May, C. E., and Rose, E. R., *J. Biol. Chem.*, 1922, liv, 213) with certain modifications.

Further investigation of the proteins of the locust bark is in progress.

THE FATTY ACIDS OF BLOOD PLASMA.

By W. R. BLOOR.

(From the Department of Biochemistry and Pharmacology, The University of Rochester, Rochester, New York.)

According to our present belief, the unsaturated fatty acids in the animal body represent stages in the oxidation of the fatty acids of the fats of the food and stores. Consequently their

occurrence, mode of combination, and relative proportions in the various tissues and organs become a matter of importance in the consideration of fat metabolism. In earlier work, data on their occurrence and distribution in the blood plasma have been reported, and the present communication is a report on attempts to determine their nature and relative proportions. The work indicated that of the fatty acids in all types of combination in blood plasma, oleic and linolic in varying proportions constitute the main constituents with palmitic acid next, then a small amount of a four double bond acid, and considerably less of an acid with three double bonds.

THE INFLUENCE OF DIET ON FAT PRODUCTION IN THE ANIMAL BODY.

By WILLIAM E. ANDERSON.*

(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)

We have approached the problem of fat production in the animal body by means of the more recent methods of animal feeding, by which due consideration can be given to each of the following factors: (1) total calorific value of ration; (2) variations in the protein with respect to its biological "quality," and the plane of concentration in the ration at which it is fed (this involves the so called nutritive ratio); (3) content and character of the carbohydrates fed; (4) fat factors furnished preformed in the diet; (5) the mineral nutrients with particular reference to deficiency of any individual element; and (6) the indispensable vitamins.

In order to determine the nature of the fat deposited under different conditions of "controlled" diet and to determine something regarding its chemical character, the fat is rendered from the entire eviscerated animal.

Peanut oil, cottonseed oil, soy bean oil, corn oil, cocoanut oil, Crisco, lard, and butter fat, which represent oils and fats of a widely varied character, have been fed in different diets, and the quality of the resulting body fat has been examined in rats ranging in weight from 140 to 250 gm. The results obtained thus far

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have clearly shown the specific influence of the different oils and fats fed, as judged by the variations in the iodine number and refractive index values.

By way of contrast the effect of a diet rich in carbohydrate and containing less than 1 per cent of fat has been studied. Both young and mature animals raised on this diet yield a comparatively hard fat. Furthermore, when the diet of rats raised to 135 and 150 gm. is changed from one containing peanut oil, cottonseed oil, soy bean oil, and corn oil to this high starch diet, mature rats produce fats which compare very closely in texture with the fats obtained from rats fed on the high starch diet. When a diet high in protein is fed, the fat produced is very similar to that laid down on the high carbohydrate diet.

THE FATTY ACIDS EXCRETED BY DOGS ON A FAT-FREE DIET.

By W. M. SPERRY.

(From the Department of Biochemistry and Pharmacology, The University of Rochester, Rochester, New York.)

In connection with a study of the lipoids excreted by dogs on a fat-free diet during a period of 5 weeks, the fatty acids were separated and their composition determined. Despite the fact that the composition of the volatile acids seemed fairly constant, averaging about 65 per cent acetic, 20 per cent butyric, and 15 per cent caproic when analyzed by the Duclaux method as modified by Gillespie and Walters, no direct relationship could be shown between the amounts of the volatile and non-volatile fractions. Moreover, no connection was observed between the amounts of the non-volatile fractions and the weights of the animals. Although the dogs varied between 5.3 and 12.9 kilos in weight, the non-volatile fatty acid fraction averaged 1.012 gm. per dog per week with a maximum variation of 0.641 gm. and an average variation of 0.216 gm. The liquid acids were always excreted in greater amount than the solid, there being in most cases $1\frac{1}{2}$ to 2 times as much of this fraction present. Separation and analysis of the bromides indicated that the liquid acids consisted almost entirely of an acid with one double bond with small amounts of an acid with three double bonds always present.

THE FORMATION OF LACTIC ACID BY THE DIABETIC ORGANISM.

By EDWARD A. DOISY, A. P. BRIGGS, C. H. WEBER, AND IRENE KOECHIG.

(From the Laboratories of Biological Chemistry of St. Louis and of Washington Universities, St. Louis.)

In agreement with the results of Barr and his collaborators on phlorhizinized dogs, we find that depancreatized dogs form large amounts of lactic acid during muscular exercise. Our data indicate that as long as the muscles contain glycogen, lactic acid is formed during contractions.

To supplement these data we have studied the chemical changes in the stimulated muscles of phlorhizinized frogs. Preliminary results on the mechanism of contraction indicate a normal utilization of carbohydrate or lactic acid in the oxidative recovery. The percentage of total fatty acids was not altered.

**TYPES OF TOLERANCE FOR DIFFERENT CARBOHYDRATES
(GALACTOSE, PENTOSE).**

By HILDING BERGLUND AND TSANG G. NI.

(From the Department of Biological Chemistry, Harvard Medical School, Boston.)

The normal tolerance for galactose after oral administration to man shows great individual variations unknown for glucose or fructose. The degree of galactose tolerance in a given individual is constant when tested on different occasions. Above the limit of tolerance, rapidly increasing loss of galactose occurs with increasing amount taken.

The behavior of pentoses is entirely different from that of the hexoses. The percentage lost in the urine seems to be essentially constant, independent of the amount injected.

THE EFFECT OF FASTING UPON CARBOHYDRATE UTILIZATION.

By ELMER L. SEVRINGHAUS.

(From the Department of Physiological Chemistry, University of Wisconsin, Madison.)

In the first 2 hours after an ordinary meal there is first a moderate hyperglycemia, then a return to normal blood sugar concentration. In many individuals there is immediately following

this a hypoglycemia of varying degree and duration. Usually there is a final return to the "fasting" level in about 2 hours after the meal. Some persons show a delayed relief from hypoglycemia, and a corresponding marked hunger. Hunger is not correlated with any definite blood sugar level, or necessarily with a subnormal blood sugar concentration. If the same meal is given three times daily, the reaction to each meal is similar. No difference is observed when a fast of 24 to 36 hours intervenes between meals. But when the fasting is for 2 days or longer the blood sugar curve after the meal is entirely different. The hyperglycemia is more pronounced, and there is little tendency to return to normal blood sugar level in the first 2 hours. The eventual return is slow. The curve suggests the "sugar tolerance curve" of the very mild diabetic. It is interpreted as indicating delayed pancreatic response. Ketosis is found to be present when this abnormal response to the meal occurs. The shorter fast induces no ketosis. Ketosis or possibly some other product of fasting seems to inhibit either the production or the action of insulin.

THE THRESHOLD OF KETOGENESIS IN PREGNANCY.

BY VICTOR JOHN HARDING, KATHLEEN DREW ALLIN, ALFRED BLYTHE EAGLES, AND H. B. VAN WYCK.

(From the Department of Pathological Chemistry, University of Toronto, and the Metabolic Ward of the Burnside Wing, Toronto General Hospital, Toronto, Canada.)

Diets in which 74 to 78 per cent of the total calories were obtained from fat were fed to women in various stages of pregnancy. Such diets are at the threshold of ketonic production when fed to non-pregnant individuals, and controls with non-gravid women showed only small amounts of "acetone" excretion per day over a period of 8 days. Thirteen experiments on pregnant women showed excretions of acetone varying from 2 to 12 gm. of "total acetone" a day.

The composition of the burning metabolic mixture was calculated from the total calories (basal metabolism + 20 per cent increase for movements in bed and the specific dynamic action of protein), from the urinary N, and assuming the complete com-

bustion of the carbohydrate. The ketogenic-antiketogenic ratios calculated according to Shaffer¹³ showed ratios varying from 1:0.45 to 1:0.87. Under these circumstances no acetone should be formed. Reckoned according to Shaffer's earlier calculations¹⁴ the ratios lie between 1:0.86 and 1:1.38. Compared with normal non-pregnant individuals¹⁵ when on diets of similar composition the threshold of ketogenesis in pregnancy appears to be low. This is taken to be true as an average result. Until further work has been carried out on non-gravid women as controls, and under conditions strictly comparable with the experiments in pregnancy, the authors, however, hesitate to regard it as true in all cases. The factors which can influence the production of ketonuria are so many that strictly accurate comparison of the two states is only possible with a larger number of cases than those as yet studied.

THE EFFECT OF INSULIN ON THE RESPIRATORY METABOLISM.

BY WALTER M. BOOTHBY AND ROBERT WEISS.*

(From the Section of Clinical Metabolism, Mayo Clinic and Mayo Foundation, Rochester, Minnesota.)

We have carried out approximately 50 experiments on men and dogs to study the effect of insulin on the respiratory metabolism. We have confirmed the original observations of Boothby and Wilder that insulin does not increase the rate of heat production in man if given in amounts which do not decrease the blood sugar to the point where a hypoglycemic reaction is produced. On the other hand, if a reaction develops, an increase in metabolism occurs even when no movements are made and no demonstrable change in tonus occurs. The most satisfactory subject upon which to show this phenomenon is a diabetic patient with a high blood sugar. After 30 units of insulin the blood sugar of one of Dr. Wilder's patients dropped from 190 to 70 mg. per 100 cc. during 140 minutes and the heat production remained constant until the sudden development of the typical hypoglycemic reaction at 69 mg. when the fall in blood sugar suddenly stopped;

¹³ Shaffer, P. A., *J. Biol. Chem.*, 1922, liv, 399.

¹⁴ Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 449.

¹⁵ Hubbard, R. S., and Wright, F. R., *J. Biol. Chem.*, 1922, i, 361.

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with the reaction the metabolism increased. In dogs we invariably obtained an increase in heat production of 10 to 20 per cent coincident with a fall in blood sugar which would probably cause a hypoglycemic reaction. It was anticipated that the introduction of glucose through a gastrostomy wound at a rate sufficient to keep the blood sugar from dropping, would prevent the increase in heat production; such, however, was not the case in a series of six experiments although the control experiment with the same quantity of glucose alone without the insulin caused no increase.

In one dog during the course of these insulin experiments, we have been able to elevate the basal respiratory quotient by high carbohydrate diet to 1.10. Regardless of the initial level of the basal respiratory quotient the administration of insulin still further raises the quotient. It is impossible in dogs to run the blood sugar and CO_2 -combining power at the same time as the metabolism; in control experiments, however, we get in general, but not invariably, a drop of 4 to 7 volumes per cent in the CO_2 -combining power after 12 units of insulin. It can be calculated from our data for the dog that the gradual decrease during 90 minutes of 7 volumes per cent will increase the CO_2 elimination per minute by 4 or 5 cc. which with an oxygen consumption of 70 cc. per minute will elevate the quotient approximately 0.07; for example, from 0.90 to 0.97. Likewise on man, we frequently obtain a drop of 5 volumes per cent in CO_2 -combining power after insulin. It is obvious that until further quantitative relationships are established between the CO_2 -combining power and the rise of the respiratory quotient it is not justifiable to draw deductions from the respiratory quotient in regard to the effect of insulin on the metabolic processes.

THE INFLUENCE OF INSULIN, ADMINISTERED ORALLY AND SUBCUTANEOUSLY, IN PHLORHIZIN DIABETES.

By O. H. GAEBLER.

(From the Department of Vital Economics, The University of Rochester, Rochester, New York.)

The influence of insulin introduced subcutaneously and orally into phlorhizinized dogs has been studied in twelve subjects. Phlorhizin was administered by the Coolen method (in olive oil)

once or twice daily. All urine periods were separated by catheter. Sugar was determined by Benedict's method, and nitrogen by the Kjeldahl-Gunning method. Blood sugars were taken only occasionally and were determined by the Folin and Wu or by the Hagedorn-Jensen method. The respiratory metabolism was followed in seven subjects by means of a closed circuit apparatus, including a chamber of the Benedict type. The insulin was made in this laboratory by the rapid method, *i.e.* amyl alcohol precipitation, and for oral administration was made into enteric coated tablets or pills from already mixed material supplied by the Sharp and Dohme laboratories in Baltimore, or was combined as desired in the laboratory with various substances which might be supposed to delay the destructive action of trypsin. Two animals only of the twelve were fed on a regular diet of lean beef heart. Dogs 6 to 11 inclusive were given glucose (Merck's c.p. dextrose) in exact amounts every day by stomach tube. The effects of insulin on the hourly excretion, as well as total for 24 hours, of dextrose and nitrogen were studied simultaneously with the respiratory quotient.

CONCLUSIONS.

1. Insulin introduced subcutaneously into dogs completely under the influence of phlorhizin acts immediately to cause a reduced rate of excretion of sugar, and an increase in the respiratory quotient.

2. When glucose is given the rate of excretion of nitrogen is reduced (protein is spared), but when glucose and insulin both are given it is reduced still more. These two fractions represent the "dextrose" and "complementary nitrogen" of A. I. Ringer.

3. Insulin administered orally in enteric coated tablets, combined with malic acid, sodium oleate, and an amino acid which liberates HCl, has given positive results on the reduction of urine sugar, increase in the R. Q., and sparing of protein. The effect is much less in amount than from subcutaneous administration.

4. The sugar which disappears from the urine and does not reappear may amount to from 0.28 to 0.48 gm. per clinical unit (1/3 of "Rochester Unit") of insulin administered subcutaneously.

No exact estimate could be made after oral administration, but, of course, it is very much less than after subcutaneous use.

5. Some of the sugar which disappears from the urine may reappear when the insulin effect wears off, because, as Cori has shown for the rabbit, it may be stored temporarily as glycogen.

THE INFLUENCE OF INSULIN AND EPINEPHRINE ON THE LACTIC ACID CONTENT OF BLOOD AND TISSUES.

By CARL F. CORI.

(From the State Institute for the Study of Malignant Disease, Buffalo.)

In view of the importance of lactic acid as an intermediary product of carbohydrate metabolism an investigation of the influence of insulin and epinephrine on the lactic acid content of blood and tissues was undertaken. The experiments were carried out on rabbits, cats, on one dog, and on mice. The mice were used for the determination of the lactic acid in the tissues, while the changes in the lactic acid of the blood were followed in the other animals.

The normal lactic acid content for rabbits (twelve experiments) varied from 13.2 to 39.7 mg. per 100 cc. of blood. Insulin given to fasting rabbits, to fasting and phlorhizin-poisoned rabbits, and insulin followed by glucose in order to relieve hypoglycemic symptoms, did not produce a significant change in the lactic acid content of the blood. However, a few minutes after a strong convulsive seizure high lactic acid values up to 106 mg. per 100 cc. of blood were found. Epinephrine caused in rabbits a decided rise in the lactic acid content of the blood. The normal lactic acid values for cats (fifteen experiments) varied from 15.1 to 34.1 mg. per 100 cc. of blood. Insulin was given to fasting normal cats, to fasting depancreatized cats, or insulin was followed by glucose in order to relieve hypoglycemia, but no decided change in the lactic acid content of the blood was recorded. Epinephrine caused an increase in the lactic acid content of the blood, but the response was not as constant nor as marked as in rabbits.

The lactic acid content of the liver and the muscles of mice was determined during moderate insulin hypoglycemia, during insulin convulsions or coma, and during epinephrine hypoglycemia. A control mouse was always killed as simultaneously as possible

with an injected mouse. There was no decided difference between the lactic acid content of the tissues of the control animals and of the injected animals. Glycogen and lactic acid maximum determinations showed that even during insulin convulsions there were enough reserve carbohydrates present in the liver and the muscles to give much higher lactic acid values than were actually found.

AN INTERPRETATION OF THE BIOLOGICAL REDUCTION OF METHYLENE BLUE.

By W. MANSFIELD CLARK, BARNETT COHEN, AND H. D. GIBBS.

(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)

The view is advanced that the reductive process of the cell is a unique characteristic of life for the study of which there still have to be developed adequate concepts, reliable methods of investigation, and sets of exact quantitative data.

This contribution is the establishment of equilibrium values for the reversible reduction of the most widely used indicator, methylene blue.

It is also established that the two electrochemical equivalents concerned in the oxidation-reduction process are associated with exactly the same energy intensity, and that the hydrogens entering into the ordinary formalistic equation of reduction are associated with ionization constants of enormous differences. This is inconsistent with Wieland's theory.

While no conclusive proof of mechanism is established it may be inferred that the reduction of methylene blue consists of the transfer to this oxidant of an electron pair. This may or may not be followed by one or the other components of water according to the acid-base equilibrium state of the solution.

Electrode potential measurements with cell suspensions (bacteria and liver) give data agreeing with those calculated from the data for the reduction of methylene blue.

The potentials thus established by two methods lie in a zone where there can be no appreciable quantity of either molecular hydrogen or molecular oxygen in *equilibrium* with the system.

If either gas be present it can be considered only in relation to a dynamic process and not to a static equilibrium.

A STUDY OF ALL-DAY BLOOD SUGAR CURVES IN NON-DIABETIC INDIVIDUALS AND IN DIABETIC PATIENTS TREATED WITH AND WITHOUT INSULIN.

BY LEON JONAS, T. GRIER MILLER, AND IDA TELLER.

(From the William Pepper Clinical Laboratory, University of Pennsylvania, Philadelphia.)

As a result of the study of thirty-four curves plotted to show the concentration of the blood sugar at frequent intervals over varying periods of time in both non-diabetic and diabetic individuals on various diets it is concluded: (1) that the dosage of insulin and the time of its administration to patients with diabetes must be controlled by a consideration of the concentration of the blood sugar throughout the entire day; (2) that a curve showing roughly this full day concentration can be constructed in most cases by having the patient on a diet of equal value as to the protein, fat, and carbohydrate content at each of his three regular meals and by determining the blood sugar level before breakfast, or before the morning administration of insulin if that is being employed, an hour after breakfast, and just before luncheon; and (3) that the ideal method for the management of such cases has not yet been arrived at, but that the following general rules for the administration of insulin to those patients with diabetes who require it seem warranted.

With a maintenance diet equally distributed among the three meals of the day mild cases may be kept within the normal limits of glycemia by means of a single dose administered a half hour before breakfast.

More severe cases require in addition to an adequate morning dose a second but somewhat smaller one a half hour before the evening meal.

When the morning fasting level of blood sugar cannot be kept below the threshold value for glycosuria by these two doses a third one is indicated at about midnight.

OBSERVATIONS ON BLOOD PHOSPHATES AS RELATED TO CARBOHYDRATE METABOLISM.

BY A. BOLLIGER AND F. W. HARTMAN.

(From the Laboratories of the Henry Ford Hospital, Detroit.)

The inorganic phosphates of the blood are depressed during carbohydrate metabolism if insulin is available. This is best shown in the comparative studies on normal, partially depancreatized, and completely depancreatized dogs.

The earliest defect in pancreatic function is demonstrated in a retarded but prolonged depression of the curve of inorganic blood phosphates. The complete absence of functioning pancreas is shown by the blood phosphate level which remains unaffected as a straight line during carbohydrate metabolism.

Carbohydrates and phosphates are best utilized when there is an excess of each in the circulation.

Medullary puncture and epinephrine effect a hyperglycemia with a corresponding fall in the inorganic blood phosphates.

As opposed to epinephrine, pituitrin produces an immediate and sharp increase of the blood phosphates along with the usual hyperglycemia. This gives additional support to the view that stores of carbohydrates and phosphates are actively broken down.

THE DISTRIBUTION OF THE UNSATURATED FATTY ACIDS, CHOLESTEROL, AND CHOLESTEROL ESTERS IN EXPERIMENTAL ANEMIA.

BY MEYER BODANSKY.

(From the Laboratory of Biological Chemistry, University of Texas, Galveston.)

In acute experimental anemia due to acetylphenylhydrazine or symmetrical diisopropylhydrazine, the total cholesterol in the plasma is not markedly affected, while in the corpuscles it is usually increased. Cholesterol esters are absent from the corpuscles of normal dogs, but appear in relatively high concentration in the corpuscles of anemic dogs. Of the total cholesterol in the corpuscles, as much as 45 per cent has been found in the form of esters. Cholesterol esters in the corpuscles have been observed, likewise, in the mild anemia which follows splenectomy.

The concentration of the unsaturated fatty acids in the plasma and whole blood remains within physiological limits in experimental anemia, but in the corpuscles, the concentration may be as much as doubled. The altered content of the unsaturated fatty acids was most marked when the anemia was associated with an increased volume and decreased hemoglobin concentration of the individual corpuscles. The presence of cholesterol esters and of increased amounts of unsaturated fatty acids indicates that the individual corpuscle in anemia may be concerned with a proportionately greater amount of lipid transformation than normally.

It does not appear that the unsaturated fatty acids play any significant rôle in blood destruction. This is made evident especially in symmetrical diisopropylhydrazine anemia. The initial effects are severe fatty degeneration of the liver accompanied by anhydremia, the anemia developing subsequently. Even during the height of the intoxication, no significant variations from the normal concentrations were observed in the case of the unsaturated fatty acids of the blood.

CUMULATIVE TESTS OF THE POSSIBLE TOXICITY OF INTARVIN, WITH NOTES ON ITS UTILITY IN THE TREATMENT OF DIABETES.

By HATTIE L. HEFT, MAX KAHN, AND WILLIAM J. GIES.

*(From the Laboratories of Physiological Chemistry at Teachers College,
and of Biological Chemistry at the Beth Israel Hospital, and the School
of Medicine of Columbia University, New York.)*

Two series of experiments on albino rats, through successive generations, have been conducted to determine whether intarvin, added to a *natural* balanced diet, in quantities ranging from 5 to 12 per cent of the total daily food intake, manifests toxicity. Eight normal female rats from one litter were originally separated into groups of four, and one of two males of practically the same size and vigor from an unrelated litter was added to each group. One such group received the balanced diet with a definite proportion of intarvin; the other received the balanced diet, with rendered lamb fat instead of intarvin added to it. In each successive generation, thereafter, the maternal rats that received intarvin

were selected from the direct descendants of those that had been receiving the "intarvinated" diet. The control maternal rats were direct descendants of those that received a diet containing an addition of lamb fat. The paternal rats were not related to the prospective mothers, but were selected on the same dietary basis as that for the maternal, or from litters on the balanced diet without addition of fat.

Rats have already been carried, in this parallel manner, into the seventh generation. There have been no discernible effects on the animals thus treated, nor on their fecundity, at any stage of the tests. Similar experiments, with intarvin the only fatty ingredient of synthetic diets, are under way.

Further study of the beneficial effects of intarvin in the treatment of diabetes has fully confirmed the earlier indications of its utility and its lack of toxicity, even when eaten to excess. It has been fed to patients daily for more than 2 years, and has induced neither gastrointestinal derangement nor other indications of toxicity in any of them. It is absorbed to the extent of 95 per cent of the amount eaten; it is not only non-ketogenic but sometimes acts as an antiketogenic agent; and its metabolic products never increase the general acidosis of the organism.

After eating intarvin continuously, in adequate amounts, diabetic patients gain in weight and strength, and lose their sense of hunger. Intarvin complements the action of insulin, and often reduces the dosage of insulin. Intarvin may be used advantageously in diabetes, whether the patient receives insulin or not. The dietetic minimum of intarvin in diabetes is the quantity that will meet the caloric maintenance requirement. Further clinical study is actively in progress.

THE CHEMICAL AND BIOLOGICAL PROPERTIES OF PURE ARSPHENAMINES.

By PHILIP ADOLPH KOBER.

(*Nepera Park, New York.*)

The elimination of methyl alcohol in arsphenamine, using the hydrochloric acid method described by the author in 1919, produces a uniform product of high purity, containing 0.3 per cent of sulfur, a trypanocidal power of about 8.5 mg. per kilo, with

a toleration of about 190 mg. per kilo, light yellow in color, non-hygroscopic, with strong gel-forming tendency, easily soluble in warm water, and insoluble in methyl alcohol. Most arsphenamine and salvarsan preparations of to-day are of the type easily soluble in cold water and in methyl alcohol, distinctly different from Ehrlich's first preparation and from pure arsphenamine. Laboratory evidence indicates that these easily soluble types may be as much as 50 per cent less potent than a pure product. Chemical evidence supports the theory that this change of character is due to the methyl alcohol in the final product having combined with the base as a methyl derivative of some sort. Reprecipitation of these methyl alcohol arsphenamines with hydrochloric acid raises in most preparations their potency and lowers their toxicity, and also their sulfur content.

Pure neoarsphenamine, having 30 to 31.5 per cent of arsenic depending on the amount of water of crystallization left in it, yellow to orange color depending on the size of crystal, is very much like pure arsphenamine: (1) slowly but not difficultly soluble in water, with some gel formation; (2) a like trypanocidal power of about 9.0 mg. per kilo; and (3) toleration in rats approximately equal to that of arsphenamine, 185 mg. per kilo. Neoarsphenamines in this country as in England in 1922 were made so as to be administrable in concentrated solutions without clinical reactions; *i.e.*, very mild. In common with the original neosalvarsan these neoarsphenamines have only 18 to 20 per cent of arsenic and contain approximately 40 per cent of impurities. These impurities do not, as does methyl alcohol in arsphenamine; increase the toxicity, but on the contrary, decrease it. This decrease in toxicity may be as much as 300 per cent. The result of making the neoarsphenamines innocuous is likewise to diminish the curative power, as much as 300 per cent and more. In other words, the character of the drug has been changed.

These mild arsphenamines, made not for their purity, but because they are, particularly the neoarsphenamines, the most innocuous, are such deviations from the curative essence or base, that it is a fair question whether or not the Public Health Service, whose regulations pay no attention to chemical purity or potency, should not call a halt to the manufacture and sale of such semi-fraudulent preparations, or insist on their being accurately labelled

or whether the trustees of the basic patents would not be justified in withdrawing the license from those whose use of the patents results in products so different than the products intended by the inventor and so contrary to public health interests.

All chemical and biological evidence proves that arsphenamines themselves, and not their impurities only, have a certain toxic power as well as a certain curative effect, and any attempts to deny this or to alter these properties in these drugs, are as reasonable as to attempt to take the poisonous qualities out of strychnine or any other powerful drug. If milder derivatives are found to be useful, let them be called and treated as new products, which in truth they are.

**THE RELATION BETWEEN THE UNDETERMINED NITROGEN OF
THE BLOOD AND ITS TOXICITY TO LUPINUS ALBUS
SEEDLINGS.**

BY JOSEPH M. LOONEY AND DAVID I. MACHT.

*(From the Biochemical Laboratory of the Sheppard and Enoch Pratt
Hospital, Towson, and the Pharmacological Laboratory of Johns
Hopkins Medical School, Baltimore.)*

In a recent communication by one of us¹⁶ it was shown that the undetermined nitrogen was increased in the blood of markedly depressed patients. This was taken as an indication of the presence of toxic amines.

While this work was in progress there was published by the other author¹⁷ a paper in which it was demonstrated that there was present in menstrual serum a toxin that decreased the growth of *Lupinus albus* seedlings in Shive solution from 75 to 51 per cent.

It was thought that the blood of these psychotic patients might be tested for the presence of toxic products in a similar manner. We have estimated the undetermined nitrogen and the coefficient of growth of the *Lupinus albus* seedlings in the blood of a number of patients.

The averages of these figures for nineteen toxic and eight non-toxic cases are given in Table I.

¹⁶ Looney, J. M., *Am. J. Psychiat.*, 1924, iv, 29.

¹⁷ Macht, D. I., and Lubin, D. S., *J. Pharmacol. and Exp. Therap.*, 1922, xxii, 413.

TABLE I.
Blood from Psychotic Patients.

	8 cases non-toxic to <i>Lupinus albus</i> .	19 cases toxic to <i>Lupinus albus</i> .
Undetermined nitrogen (a), per cent non-protein N.....	14.0	19.5
Index of growth (b), per cent.....	75.0	53.5
Factor ($a \times b$).....	1,050	1,040

The third line gives the factor resulting from multiplying the percentage of undetermined nitrogen by the percentage of growth for both groups. The constancy of this factor is quite striking and offers very remarkable support to the idea that the toxicity of the bloods varies with the undetermined nitrogen.

It is to be noted that this toxin is evidently very different from that found in menstrual serum which is possibly due to a derivative of cholic acid,¹⁸ as the specimens taken were from women who were not menstruating or who had passed the menopause and from men; some of the most toxic specimens being from men.

A STUDY OF THE ENERGY AND SUBSTANCE METABOLISM OF UNDERNOURISHED CHILDREN.*

By CHI CHE WANG, RUTH KERN, MARGARET FRANK, AND JEANNETTE DUNWIDDIE.

(From the Nelson Morris Memorial Institute for Medical Research,
Michael Reese Hospital, Chicago.)

Thirty-one undernourished children, ranging in age from 4 to 13 years, served as subjects under well controlled conditions. They were divided into three groups based on percentage underweight according to their height, varying from +6 to -26 per cent. Four of them were slightly above normal height, two exactly normal, and the other twenty-five were under height, ranging from 1 to 15 cm. below normal. All the children looked pale and sickly.

¹⁸ Macht, D. I., and Hyndman, O. R., *J. Pharmacol. and Exp. Therap.*, 1924, xxii, 483.

* Aided by a fund contributed jointly by the Elizabeth McCormick Memorial Foundation, Mrs. Francis Nielson, and Mrs. Gusta Morris Rothschild.

Basal metabolism was determined by means of the Benedict chamber bed and Universal respiration apparatus. There is no difference in the basal metabolism of the three groups, computed on the basis of total calories or surface area referred to either age or weight, but in all these cases the figures were higher than the Benedict standards. An interesting observation was that the basal metabolism of these children was comparable with that predicted for children of their age who were normal in height and weight.

The energy value of excretions was determined by means of Benedict's bomb calorimeter. The total loss varied from 108 to 199 calories per 24 hours, but here again there is no difference in the three groups. On the whole, the most underweight children had a higher per kilo intake and hence a higher caloric utilization per kilo of body weight. The calorie:nitrogen ratio in all groups ranged between 7.1 and 12.2, which is within normal range according to Uthelm.

The per kilo nitrogen intake, in agreement with the caloric intake, shows very little difference in the three groups. The average of each group is, however, higher in the more undernourished children, being 0.431, 0.465, and 0.514 gm., respectively. The average value for absorption per kilo of body weight follows in the same manner, namely 0.386, 0.413, and 0.466, but the nitrogen retention is only 0.069 gm. in the third group as compared with 0.087 and 0.089 gm. in the other groups. This tends to indicate that food is not so well digested by the more undernourished children, but more is excreted through the feces.

Calcium metabolism shows no difference among the three groups, in intake, output, utilization, or retention.

All the children showed a creatinuria, that of the first and second groups averaging 7.5 and 7.6 mg. per kilo, and that of the third group 9.2 mg. per kilo. On the other hand, creatinine per kilo is practically constant in the three groups, averaging 20.6, 21.9, and 21.7 mg. per kilo, respectively. The creatinine coefficient is 7.9 for the three groups, which is comparable with reported figures for adults. Ammonia nitrogen was well within normal limits in all cases, as was also the ammonia nitrogen:total nitrogen ratio.

The lack of variation from normal may be due to the fact that

these children are not sufficiently undernourished to be really emaciated. Only three cases are more than 20 per cent underweight. The likelihood is that they are deficient simply in their fat stores, and that their actual tissue metabolism still approximates that of normal children.

THE INFLUENCE OF INFECTION ON THE LIPOIDS OF THE SUPRARENAL GLAND.

BY EMIL J. BAUMANN AND OLIVE M. HOLLY.

(From the Division of Laboratories, Montefiore Hospital, New York.)

Normal suprarenals were compared, in regard to cholesterol, phosphatide, and fat, with the suprarenals taken from animals that died, or were killed, because of infections.

In the suprarenals of normal guinea pigs the average "fat" (total ether-alcohol-soluble matter less cholesterol and phosphatides), cholesterol, and phosphatide content was 7.7, 3.7, and 1.8 per cent, respectively, while in animals that died of various infections the corresponding average values were 11.2, 1.0, and 2.2 per cent. The most characteristic and uniform difference is the marked loss of cholesterol.

Similar observations were made on rabbits. Unlike the uniform figures found for guinea-pigs, the variations among normal animals were very large. This can be explained in part by the fact that the stock had been exposed to snuffles, although in the animals used for "normals" no nasal mucous discharge had been noted during the period of observation. In spite of this very serious complicating factor several deductions may be made from these data.

The average "fat," cholesterol, and phosphatide percentages of the suprarenals of twenty-six normal rabbits were, respectively, 13.2, 8.8, and 2.5 per cent. The corresponding values in the case of nine rabbits that died of pneumonia, peritonitis, etc., were 13.8, 4.8, and 2.8 per cent. Thirteen rabbits, sacrificed because of obvious illnesses, which proved on necropsy to be similar infections, show averages of 16.0, 11.1, and 2.2 per cent, respectively.

It would appear that during the early stages of these infections an *increase* in the cholesterol content of the suprarenal occurs,

which is followed by a decrease later in the disease. The fact that a rise of the percentage of cholesterol occurs is more strikingly brought out by the figures obtained from analyses of the suprarenals of four rabbits that were sacrificed because of middle ear infections—comparatively a very mild and localized infection. The average figures for “fat,” cholesterol, and phosphatide were 8.5, 20.6, and 2.6 per cent, respectively.

ON SPECIFIC SPERM AGGLUTININS.

By G. H. A. CLOWES AND EDA B. WALDEN.

(From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.)

The substance derived from sea urchin eggs by aqueous extraction, which is capable of exerting a specific agglutinating action on sea urchin sperm, may be profoundly modified as regards its susceptibility to heat and various chemical reagents by extracting certain lipoids from the solutions containing the agglutinin or by treating the eggs in advance with appropriate concentrations of certain organic reagents.

These results harmonize with those previously observed by the writer regarding the influence exerted by lipoids on the heat sensitivity of pollen toxins, and appear to throw some light on the physical constitution of protoplasmic particles.

A COLORIMETRIC METHOD OF DETERMINATION OF BILE SALTS IN THE BLOOD.

By SHIRO TASHIRO.

(From the Biochemical Department, University of Cincinnati, Cincinnati.)

Pettenkoffer's method of detection of bile salts is so regulated that the color developed is permanent enough to give ample time for colorimetric estimation. With a proper process of extraction, this method is sensitive to measure bile salts in blood in a dilution as small as 5 in 100,000. A simple method of preparing permanent standards, which give a perfect match to the color developed with Pettenkoffer's method with the bile salts, will be given.

**THE COLORIMETRIC DETERMINATION OF TRYPTOPHANE BY
THE VANILLIN-HCl REACTION AND THE QUANTITATIVE
SEPARATION FROM INDOLE AND SKATOLE.**

By IDA KRAUS.

*(From the Hull Laboratories of Physiological Chemistry, University of
Chicago, Chicago.)*

The color reaction here employed distinguishes indole and skatole from tryptophane by the differences in the colors obtained. Indole and skatole can be removed quantitatively by extraction with toluene before the reaction is applied. Tryptophane can thus be recovered quantitatively. Hydrolysis of a protein by barium hydroxide not only destroys tryptophane, but also brings about other decompositions which alter the aldehyde condensation reactions. Barium hydroxide hydrolysis of proteins and of tryptophane respectively produces different decomposition products which are reactive with tryptophane reagents. These products are precipitated by mercuric sulfate. The barium hydroxide hydrolysis of pure tryptophane actually causes a loss of 7 to 20 per cent thereof, depending on whether the phenol reagent of Folin and Looney or the vanillin-HCl reagent is employed in the quantitative estimation. At present trypsin hydrolysis of proteins is the most satisfactory procedure for the estimation of tryptophane in proteins. By the method here developed we find 1.26 per cent in casein, 0.53 per cent in U.S.P. pancreatin, 0.19 per cent in zein, and 0.12 per cent in gelatin. The value found for casein agrees very favorably with the assumptions that the molecular weight of casein is of the order of 16,000, and that 1 molecule of tryptophane is present. The low values for zein and gelatin suggest molecular weights of the order of 106,000 and 120,000, respectively, or that the tryptophane is present as an impurity in the form of another protein. Pure tryptophane, or tryptophane and monoamino acids, when incubated with trypsin, is recovered quantitatively by the vanillin-HCl method, but when incubated therewith in the presence of the diamino acid fraction or glucosamine a loss of 10 to 20 per cent is observed. Without incubation this loss is not observed.

ON THE RELATION OF THE THYROID TO THE EFFECTS OF INSULIN.

BY AARON BODANSKY.

(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca.)

The author has reported the typical time relations of the blood sugar curves obtained after intravenous injection of insulin in normal and thyroidectomized sheep, and in normal human subjects. His findings on the effect of thyroidectomy on the reaction to insulin have been confirmed by Ducheneau and Burn and Marks.

In an effort to establish more rigorous quantitative criteria for the reaction of the athyroid, hypothyroid, and hyperthyroid, as well as of the normal subject, to intravenous injections of insulin, the author has attempted to ascertain the conditions under which practically constant blood sugar is obtained in sheep, and a practically constant response to injections of insulin, and has repeated his experiments under these conditions.

FURTHER STUDIES ON THE EFFECT OF FINE GRINDING UPON STARCH GRAINS.

BY C. L. ALSBERG AND E. E. PERRY.

(From the Food Research Institute and the Department of Chemistry, Stanford University, California.)

We have shown recently that cold water extracts considerable material, giving a blue color with iodine from starch grains ground in a pebble mill until the individual grains are broken or battered, and that a suspension of such ground starch in water is incapable of gelatinizing or yielding a paste when heated (except in concentrations of 10 to 15 per cent by weight). It can now be reported that such battered starch grains are still birefringent, though they no longer show the black cross under the polarizing microscope. They swell instantaneously when they come in contact with cold water and to a considerable degree disperse themselves in it. They stain instantaneously in aqueous solutions of a number of dyestuffs as well as being dispersed in them. Uninjured starch grains are stained with the greatest difficulty. Solu-

tions prepared by extracting ground starch in water at room temperature deposit on standing a flocculent precipitate insoluble in cold water. However, not all the soluble material is deposited. The solution after weeks of standing still contains about 0.5 per cent of material which colors blue with iodine. It is precipitated from its solutions by alcohol and the precipitate can be redissolved in cold water if it has not been permitted to become dry.

A MODIFICATION OF THE METHOD OF PREPARING GLIADIN.

BY D. B. DILL AND C. L. ALSBERG.

*(From the Food Research Institute and the Department of Chemistry,
Stanford University, California.)*

The modification consists essentially of two procedures. The first is that after the first alcoholic extract of gluten has been concentrated to a thick sirup under diminished pressure and the gliadin has been precipitated with water containing a little sodium chloride, it is redissolved in warm 70 per cent alcohol and the solution is allowed to stand in the refrigerator overnight. In this manner a gelatinous mass—impure gliadin—settles out which carries down with it nearly all the contaminating lipid material. This is otherwise very difficult to remove and interferes with clear filtration. The gliadin is precipitated with strong alcohol out of the clear filtrate after evaporation to small volume, whereupon it is again subjected repeatedly to the processes above described. In the final precipitation with water lithium chloride is used instead of sodium chloride; and this is the second modification of Osborne's method of preparation proposed in this paper. Lithium chloride has the advantage over sodium chloride where precipitation with alcohol is unsatisfactory unless an electrolyte be present, that it is freely soluble in alcohol and ether and may thus be more easily removed again.

Five preparations were made from three different flours. Their nitrogen contents (Kjeldahl) on the dry basis are 17.55, 17.53, 17.53, 17.56, and 17.49 per cent. Their ash contents are 0.06, 0.11, 0.07, 0.07, and 0.08 per cent. Loeb, C. R. Smith, and others consider gelatin prepared by washing at the isoelectric point and still containing nearly 0.1 per cent ash, as ash-free.

THE SPECIFIC ROTATION OF GLIADIN IN EtOH-H₂O SOLUTIONS.

BY D. B. DILL AND C. L. ALSBERG.

(From the Food Research Institute and the Department of Chemistry,
Stanford University, California.)

We have determined the specific rotation of five samples of pure gliadin, obtained from three different flours. $[\alpha]_D^{20}$ for gliadin dissolved in 50 per cent alcohol was found to be -90.0° ; in 60 per cent alcohol, -91.0° ; in 70 per cent alcohol, -90.0° ; in 80 per cent alcohol, -90.2° . Freshly prepared solutions were found to have a specific rotation about one degree greater (arithmetically) than those noted above. Our solutions were prepared by weighing the gliadin in 25 cc. volumetric flasks, adding 20 cc. of alcohol, and warming at 40° until solution was complete. The flasks were then cooled to 20° , the contents made up to volume, and polarizations made. From 12 to 48 hours later second readings were made with the results indicated above. It is believed that there is some lag in the return of the specific rotation of gliadin solutions to their normal value as they are cooled from 40° to 20° .

$[\alpha]_D^{30}$ for gliadin in 50 per cent alcohol is -91.4° ; in 60 per cent alcohol, -93.2° ; and in 70 per cent alcohol, -91.4° . $[\alpha]_D^{40}$ for gliadin in 50 per cent alcohol is -94.2° ; in 60 per cent alcohol, -95.8° ; and in 70 per cent alcohol, -93.4° . As these solutions were warmed from 20° to 30° and from 30° to 40° , the values for $[\alpha]_D^{30}$ and $[\alpha]_D^{40}$ were reached immediately and did not change after 30 minutes at each of these temperatures. Upon cooling each of these solutions from 40° to 30° the previous value for 30° was immediately attained except in the case of the solution in 70 per cent alcohol. Upon cooling from 30° to 20° , some time elapsed before the original value for $[\alpha]_D^{20}$ was attained.

STUDIES ON GLYCOLYSIS.

BY SERGIUS MORGULIS AND OTAKAR BARKUS.

(From the Department of Biochemistry, University of Nebraska, Omaha.)

In view of the importance attributed by the Embden school to lactacidogen as an intermediate phase in carbohydrate metabolism, we studied the changes in inorganic phosphorus of blood undergoing glycolysis. It is, of course, known that the disap-

pearance of the blood sugar is paralleled by an accumulation of lactic acid in the blood. If lactacidogen were, therefore, an intermediate step in this transformation, one would likewise expect to find a gradual rise in the inorganic phosphorus. Indeed, it is claimed by some that this is what actually occurs in the course of glycolysis. We performed experiments with bloods from various animals, which have been undergoing glycolysis at 33°C., and we followed the changes in sugar, lactic acid, and inorganic phosphorus at different intervals during the process. In these studies we found that the inorganic P invariably remained unchanged even when glycolysis has progressed very far and there has been a great accumulation of lactic acid in the system. This, however, is only true for experiments where there has been no hemolysis. Even a trace of hemolysis will cause an appreciable rise in the inorganic P value and, as the hemolysis becomes more extensive, the inorganic P value likewise increases manifold. We are led, therefore, to the conclusion that the formation of lactic acid resulting from glycolysis of blood is not associated with any alteration in the inorganic P content provided there is no hemolysis occurring at the same time. Evidence which has been offered to the contrary is unquestionably due to the fact that the effect upon the inorganic P produced by hemolysis has not been properly considered.

**THE MAXIMUM OF HUMAN POWER, AND THE FUEL OF MUSCULAR
WORK, FROM OBSERVATIONS ON THE OLYMPIC CHAMPION-
SHIP CREW OF 1924.**

BY YANDELL HENDERSON AND HOWARD W. HAGGARD.

(From the Department of Applied Physiology, Yale University, New Haven.)

Rowing in a racing shell with sliding seats probably allows a nearer approach to maximal work, by more nearly all the muscles of the body, than any other form of athletics. The eight men who rowed in the Yale University boat in 1924 demonstrated in a series of races, ranging from $1\frac{1}{2}$ up to 4 miles, that for all these distances they were able to lead any other crew in their own country. After winning the right to represent America in the Olympic games at Paris, they won in the trial heats and led by several lengths in the final race on the Seine, winning from crews

from all parts of the world and establishing a world's record for the 2,000 meter course.

During the season of training from January to June, 1924, we were fortunately able to make from time to time on five of these men determinations of the respiratory exchange and quotient, the oxygen consumption and deficit, the CO₂ output, etc. The external work was also determined by means of a rowing machine set up in the laboratory and arranged as an ergometer. Later a determination of the power necessary to drive a racing boat at various speeds was obtained by towing it by means of a power boat with the tow line fastened to a spring balance. The figures from the draw bar pull multiplied by the speed in feet per minute give the absolute net work which the crew had to do. An additional 25 per cent of external work was assumed in order to cover the energy expended in moving the slide and oar in returning to the stroke position, and was added to the results from the rowing machine and draw bar pull methods.

The data from these three methods were in general in fair agreement. They indicate that the maximal power exerted is from 0.45 to 0.55 horse power per man, or expressed in the heat equivalents, 4.8 to 5.9 calories per minute, with a total energy expenditure of 19 to 29 calories per minute, or thirteen to twenty times the basal rate. The power expressed by the smaller of each of these pairs of figures is that maintained, and is therefore approximately the maximum that a man can maintain, for 22 minutes during a 4 mile race; while the higher figures are applicable to the more intense exertion and greater speed, which are also maximal for about 6 minutes in races of about $1\frac{1}{2}$ miles or 2,000 meters. The corresponding figures for the volume of oxygen consumed per minute are 3.5 and 4 liters; the latter figure is about the limit of the transporting capacity of the lungs, blood, and heart. An oarsman exerts a power, which exceeds by 30 to 60 per cent that afforded by the oxygen simultaneously absorbed; he thus draws heavily on his credit, and incurs oxygen deficits of 4 to 8 liters or more and these deficits are repaid by the high rate of oxygen absorption for a time after the work is ended. This is in accord with A. V. Hill's conception.

The most significant result of these observations is the evidence, which they afford, in general agreement with Krogh and Lindhard, but in disaccord with the Hill-Meyerhof conception in its original

form, that in whatever proportion fat and sugar are being burned during rest just before the exercise, they are burned in nearly the same proportion to produce the energy for doing work and for recovery. Thus it is found that in these oarsmen the respiratory quotients for the work and recovery periods combined were approximately the same as those during the rest before the work. In one experiment the man had missed his breakfast and eaten nothing for 18 hours; yet he made an intense exertion, although rather disadvantageously, on a combustion almost entirely of fat from his own body. His respiratory quotient during rest was 0.75, during 3 minutes of rowing on the machine it was 0.72, and during 10 minutes rest afterward 0.73. The work done was equivalent to 6 calories per minute, the total energy expenditure 29 calories per minute; this man's weight was 180 pounds (82 kilos) and his height 6 feet, 4 inches (193 cm.). In general the observations show that much more than half the energy expended by these athletes in muscular work is drawn from fat, and much less than half from sugar. A much larger proportion of carbohydrates would probably be advantageous.

In contrast to the effects of great exertion on untrained men, there was in the members of this crew only a slight overbreathing, or sometimes practically none at all, with a correspondingly slight blowing off of CO_2 during work or afterward. Apparently some of the phenomena, especially the blowing off of CO_2 and the high respiratory quotient during and immediately after intense exertion, which are commonly explained as in accord with the Hill-Meyerhof conception of muscular contraction, are due to the stimulation of the respiratory nervous regulation by oxygen deficiency in the arterial blood rather than to displacement of carbonic acid from the blood carbonates by lactic acid.

FURTHER DATA ON THE METABOLISM OF DEPANCREATIZED DOGS KEPT ALIVE WITH INSULIN.

By I. L. CHAIKOFF, J. J. R. MACLEOD, AND J. MARKOWITZ.

(From the Department of Physiology, University of Toronto, Toronto, Canada.)

By daily feeding with raw pancreas (50 gm.) along with flesh (200 to 400 gm.) and cane-sugar (up to 50 gm.), and the injection twice daily of about 16 clinical units of insulin, dogs weighing

approximately 8 to 10 kilos can be kept in a perfect condition of bodily nutrition. One animal is still living after 15 months on this diet, and another after 9 months. Since none of the symptoms of hepatic breakdown, previously reported from this laboratory (Allan, Bowie, Macleod, and Robinson) as occurring in the case of depancreatized animals fed without raw pancreas, have supervened, it is concluded that the presence of something derived from the pancreas, either its digestive ferments or some internal secretion besides insulin, is necessary for the maintenance of normal metabolism. It is possible that the absorption from the intestine of toxic breakdown products of protein is responsible for the damage to the liver when no trypsin is present.

Various studies have been made of the chemical changes which occur in the blood and urine following discontinuance of insulin in these animals, for several days, and also of the changes produced by again giving this hormone. The main results show a remarkable correspondence in the rate at which changes occur in sugar, in acetone bodies, and in the phosphoric acid of the blood. All three come down at an equal rate after injecting insulin, but the phosphoric acid starts to return to the original level more quickly than the other two. No constant changes have been observed in the percentage of fat in the blood within several hours of the injection of insulin. The percentage amounts of ketone bodies, of sugar, and of fat are higher 3 to 5 days after discontinuing insulin in a fat depancreatized dog than in one that is thin.

CHEMICAL CHANGE IN FISH MUSCLE DURING RIGOR MORTIS.

By C. C. BENSON.

(From the Department of Food Chemistry, University of Toronto, and Atlantic Biological Station, St. Andrews, New Brunswick, Canada.)

It has been noted by von Fürth that the juice squeezed out from fish muscle contains considerable amounts of the protein which he calls "soluble myogen-fibrin" and, by a method which he describes, it is possible to determine its amount fairly quickly.

When large fishes were available, the tail parts were cut off and set in an apparatus to measure the amount of rigidity following death, and the remaining parts of these fishes were used to obtain

the muscle juice; or two fishes of similar size and weight were used—one for the measurement of stiffness and the other, for chemical study.

It was found that, when the fishes were soft, before rigor set in, the amount of soluble myogen fibrin was less than during rigidity and that the amount decreased as the stiffness passed off. The amount of this protein in the muscle juice is thus related to the condition of the muscle in regard to rigidity.

THE RELATIVE REDUCING POWER OF SOME COMMON SUGARS.

BY A. W. ROWE AND B. S. WIENER.

(From the Department of Chemistry, Evans Memorial, Boston.)

It has long been recognized that the reduction velocities of the simple sugars exhibit wide variations. Blood sugar methods are based upon incomplete reductions and consequently are most affected. Blood sugar curves produced by test meals of sugars other than glucose are never accurate and may be seriously in error. The relative reducing values compared with glucose as 100 are given in Table I.

TABLE I.

Method.....	Folin-Wu.		Lewis-Benedict.
Time.....	6 min.	12 min.	10 min.
Glucose.....	100	105	100
Levulose.....	90	90	99
Galactose.....	77	88	85
Mannose.....	55	92	100
Lactose.....	45	59	76
Maltose.....	40	50	82

Mixtures of the several sugars dissolved both in water and in blood plasma showed that the relative reducing powers depend upon the coefficient of each sugar and their relative proportions. With the disaccharides, hydrolysis is a possible factor, particularly in the picric acid method.

The application of the several coefficients to the calculation of approximate blood sugar levels is pointed out.

**DIETARY REQUIREMENTS FOR REPRODUCTION.
V. THE DISTRIBUTION OF THE REPRODUCTIVE DIETARY COM-
PLEX (VITAMIN E) IN VARIOUS VEGETABLE OILS.**

By BARNETT SURE.

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From work thus far completed the vegetable oils may be tentatively divided into three groups.

1. Those that do not even produce fertility. In this group belong commercial linseed, cocoanut, and sesame oils.

2. (a) Those oils which will produce fertility but fail in lactation. In this group belong commercial olive oil and the oil of the peach kernel; also soy bean and peanut oils directly expressed. (b) Those which produce fertility and are partly successful in lactation. In this group belongs commercial cottonseed oil.

3. Those oils which are potent both in fertility and lactation. In this group belong wheat germ, hemp-seed, and yellow corn oils, prepared by extraction with ether, acetone, or benzene.

Experiments are in progress to determine whether the reproductive complex is made up of two vitamins, one necessary for fertility, and one essential for lactation.

THE PRESENCE OF TREHALOSE IN BAKERS' YEAST.

By ELIZABETH M. KOCH AND F. C. KOCH.

A QUANTITATIVE PEPSIN METHOD BASED UPON REFRACTIVE INDEX AND MICRO KJELDAHL ESTIMATIONS.

By T. L. MEEKIN.

A METHOD FOR PURIFYING INSULIN.

By N. R. BLATHERWICK AND L. C. MAXWELL.

THE DESTRUCTION OF URIC ACID IN PERFUSION EXPERIMENTS.

By OTTO FOLIN, CECIL DRINKER, AND SIDNEY BLISS.

THE SITE OF AMMONIA FORMATION.

By OTTO FOLIN AND SIDNEY BLISS.

**THE INFLUENCE OF THE HORMONE OF THE OVARIAN FOLLICLE
ON THE BASAL METABOLISM OF THE RAT.**

By EDWARD A. DOISY AND J. O. RALLS.

**SOME RECENTLY INTRODUCED MICRO QUANTITATIVE METHODS
AND THEIR VALUE.**

By A. B. MACALLUM.

PLASMA LIPOIDS IN EXPERIMENTAL ANEMIA.

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The outstanding feature of all types of anemia is an abnormally low percentage of red blood cells, the result of either abnormally slow production, abnormally rapid destruction, or perhaps both. Relatively little attention has been paid to the first of these causes although the recent publications of Whipple (1) and coworkers have shown the importance of various factors on blood regeneration in experimental anemia. The second cause noted above—abnormally rapid destruction—has been given much greater attention by experimenters, and, correctly or incorrectly, is widely accepted as the most important agency in the production of anemia. Among the many substances which from time to time have been considered significant in red cell destruction, the hemolytic agents, and particularly the lipoids, have come in for a large share of attention. It has been found that the unsaturated fatty acids are hemolytic at least *in vitro* (2), although the results have often been disappointing when these substances or their soluble salts (soaps) have been introduced directly into the circulation. The living animal seems to be able to a very considerable extent to neutralize their effects.

Csonka (3) made examinations of the iodine-combining power (degree of unsaturation) of the fatty acids in the blood of pernicious anemia without finding exceptionally high values. He found, however, that the blood cholesterol in pernicious anemia was below normal. Believing that his results with regard to the unsaturated acids were uncertain because of the fact that the fatty acids of blood consist of an unknown and possibly variable mixture of saturated and unsaturated acids, he later (4) made an examination of the unsaturated acids separately, finding in pathological conditions, especially when the hemoglobin values were low, that the iodine values of the unsaturated fatty acids of blood were generally higher than

in normal conditions. Boggs and Morris (5) found in the anemic lipemia produced by repeated bleedings in rabbits that the blood fatty acids had a high iodine value. Dubin (6) found as a result of experimental (trypanosome) anemia in dogs that the total fat of the blood was increased, while the lecithin and cholesterol were decreased. An examination of the quantitative relations of the blood lipoids in different types of anemia by Bloor and MacPherson (7) did not lead to any definite conclusions. It was found that the lipoids were normal in amount and relations as long as the percentage of corpuscles remained above half the normal value. When the percentage fell below this level, abnormalities appeared, which in the order of their magnitude and also of the frequency of their occurrence were: (a) high fat including fatty acid in plasma; (b) low cholesterol in plasma and occasionally in corpuscles; and (c) low lecithin in plasma. Gibson and Howard (8) made a similar study of the blood lipoids in anemia and found low fat values in whole blood and plasma in severe pernicious anemia with a tendency to increase with improvement of the blood picture. They also made iodine number determinations of the fats using a micro method and found that the low values were associated with high iodine numbers. The differences were not considered enough to be an important factor in the production of anemia. They found low blood and plasma cholesterol in pernicious anemia, the values increasing as the blood was regenerated. It may be noted that iodine number determinations made on such minute amounts of material would almost certainly be low, particularly in the higher values, because of the greatly increased chances of oxidation with small amounts of material. MacAdam and Shiskin (9) found no notable differences in the blood lipoids between secondary and pernicious anemia.

Cholesterol is the only known antihemolytic found in blood. Its preventive effect on saponin hemolysis is so definite that recently a method has been proposed for the determination of cholesterol in blood based on this property (10). Its function as a general hemolytic has been generally assumed, although there is but little positive evidence in support of the assumption. Its power of forming loose combinations with the fatty acids in addition to its stable esters has been noted by Windaus (11) and is probably of importance in this connection.

An opportunity to study the blood lipoids in anemia on a fairly large scale was afforded by the work on anemia and blood regeneration now being carried on by Dr. G. H. Whipple and Mrs. F. S. Robbins of this school. In the course of the work, normal dogs were bled as frequently and largely as possible until a certain level of anemia (corpuscle volume, 15 to 20 per cent of the blood) was reached. The animals were kept at this level by bleeding at

such times as the regeneration had raised the corpuscle percentage above the determined level. There became available in this way considerable amounts of blood from animals with a marked degree of anemia, and, also for comparison, the normal blood from the same animals before the anemic condition was established. The plasma obtained from the blood by centrifugalization was treated as in earlier work on plasma lipoids (12)—precipitated with alcohol and the precipitate extracted with hot alcohol. The combined alcohol extracts were evaporated at low pressure, after which the intact lipoids were separated approximately by fractionation, and the composition and distribution of the fatty acids in the fractions studied. The lecithin fraction was obtained in the usual way by double precipitation from a concentrated ether solution with acetone. The first two of the ester fractions were obtained from the acetone-ether mother liquor by successive concentrations of the solvent. The free fatty acid fraction was then separated by evaporating the solvent completely, dissolving the residue in petroleum ether (b. p. 50–60°C.), and separating the fatty acid by mixing the solution with an equal volume of alcoholic potassium hydroxide, containing an excess of alkali and an equal volume of water. The fatty acid remained in the alkaline water layer, from which it was separated by acidification and extraction with petroleum ether. The other lipoids separated in the petroleum ether layer from which they were recovered by evaporation. They were then further fractioned (Fractions III and IV) from alcohol, Fraction IV being the final mother liquor. It was not possible to obtain a separate fraction of free cholesterol, probably because of the solubility of the cholesterol in its esters and the fact that it forms loose combinations with the free fatty acids. Digitonin might have been used for the purpose, but because of its scarcity and prohibitive cost it was not practicable for an extended series of experiments such as above. The fractions, except the lecithin, therefore, contain unknown amounts of free cholesterol, also as noted below, there is an unknown amount of fat distributed through them. The results of the examinations are contained in Table I.

Mar. 12	0.368	130	0.720	0.892	144	0.46	1.08	77	0.172	0.072	.25	0.132	0.	84	0	54
" 13	0.554	105	0.448	0.600	117	0.70	0.87	91	0.196	0.147	.22	0.112	0.	25	0	16
" 17	0.538	120	0.134	0.324	118	0.852	0.91	00	0.030	0.116	.11	0.410	0.	26	0	170
" 28	610		0.203	0.257	98	0.595	0.45	40	0.507	0.268	.06				0	473
" 29	500		0.472	0.376	150	0.312	0.32	63	0.016	0.150	.84	0.060	0.022		2	046
" 30	700		0.288	0.190	130	0.618	0.420	52	0.314	0.069		0.016	0.009		1	921
" 31	650	0.513	116	0.522	0.644	171	0.335	0.415	74	0.017	0.015	0.234	0.478	.42	0	358
Apr. 9	500	0.692	121	0.446	0.390	126	0.508	0.40	85	0.050	.05	0.264	0.646	.34	0	490
" 10	500	0.886	132	0.542	0.496	168	0.158	0.124	115	0.032	0.052	0.182	0.956	.46	0	642
" 7	575	0.595	118	0.682	0.685	109	0.308	0.274	45	0.077	0.059	0.117	0.358	.47	0	206
" 28	700	0.565	108	0.720	0.545	113	0.253	0.246	52	0.244	.35	0.050	0.271	.33	0	220
May 6	400	1.089	103	1.045	0.850	108	0.475	0.402	64	0.202	.63	0.117	0.685	.46	0	360
" 12	600			0.761	0.650	79	0.398	0.570	01	0.137	.13	0.057	0.303	.53	0	325
June 26	660	0.321	109	0.370	0.536	134	0.093	0.234	47	0.070	.08	0.029	0.109	.92		
" 30	400	1.461	126	1.140	1.175	202	0.245	0.348	45	0.462	.16	0.197	0.677	.36		
July 2	480			0.985	1.739	155	0.038	0.248	64	0.227	.22	0.063	0.225	.27		
" 14	500	0.440	152	0.390	0.774	69	0.164	0.264	32	0.240	.88	0.100	0.294	.48		
" 26	374	0.376	112	0.288	0.278	103	0.142	0.639	10	0.139	.05	0.092	0.099	.15	0	423
Sept. 5	750	0.598	106	0.474	0.641	114	0.301	0.450	36	0.047	.031	0.003	0.113	.11	1	749
" 4	325	0.135	107	0.087	0.044		0.308	0.269	28	0.280	.44	0.242	0.574	.20	0	492
" 30	970	0.510	135	0.100	0.103	113	0.241	0.194	86	0.360	.50	0.077	0.088		0	
Oct. 2		0.103	79	0.004	0.078	0.	0.264	.46	0.293	0.601	.85	0.072	0.214	.64	0	
	0		120	0	0.51		457		0.274					.29		20

Plasma Lipoids in Anemia

Volume of blood plasma used.	Leci- fract		ion II				ion III				Free fatty acids.	
	Fatty acids.	Iodine number.	Unsaponifiable.	Fatty acids.	Iodine number.	Unsaponifiable.	Fatty acids.	Iodine number.	Unsaponifiable.	Fatty acids.	Iodine number.	Weight.
cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	gm. per 1,000 cc.	Iodine number.	gm. per 1,000 cc.
Nov. 22	100 1.261	97	0.800	0.730	112	0.135	0.144	124	0.298	0.262	117	0.182
Dec. 11	100 1.063	112	0.689	0.570	126	0.270	0.210	126	0.174	0.275	103	0.163
Jan. 24	560 1.165	114	0.810	0.715	123	0.313	0.224		0.129	1.155		0.294
Feb. 12	480 0.830	68	0.121	0.185	70	0.634	0.630	124	0.240	0.202	83	0.229
Mar. 20	500 0.212	90	0.114	0.188	77	0.562	0.522	144	0.298	0.277	106	0.700
" 21	470 0.318	60	0.134	0.177	88	0.475	0.528	122	0.083	0.145	95	0.356
" 24	475 0.114	83	0.211	0.173	80	0.712	0.740	119	0.223	0.205	112	0.411
Apr. 8	370 0.955	111	0.108	0.141	124	0.829	0.594	136	0.028	0.231	137	0.330
June 19	350 0.754	79	0.754	0.600	131	0.921	0.255	111	0.028	0.231	137	
" 25	285 0.870	78	1.050	1.030	129	0.350	0.245	136	0.287	0.895	115	
" 2	410 0.798	76	0.895	0.770	138	0.455	0.166	119	0.136	0.223	109	
July 9	323 0.251	91	0.710	0.476	164	0.186	0.186	147	0.125	0.152	115	0.359
" 15	380 0.731	106	0.286	0.259	123	0.104	0.371	112	0.151	0.169	104	0.450
" 23	326 0.895	98	0.384	0.446	108	0.212	0.571	100	0.406	0.273	134	0.277
Sept. 4	600		0.207	0.157	106	0.360	0.283	142	0.027	0.040		1.090
" 5	670 0.272	103	0.660	1.046	126	0.269	0.491	132	0.148	0.311	136	0.886
Aug. 27	1,700 0.128	99	0.190	0.161	92	0.368	0.476	137	0.183	0.249	82	0.345
" 28	1,300 0.056		0.106	0.069		0.178	0.274	134	0.145	0.225	127	0.091
Sept. 29	1,150		0.084	0.064		0.572	0.385	144				
Average.	0.595	97	0.436	0.419	111	0.415	0.385	133	0.202	0.354	111	0.410
									0.159	0.389	102	

DISCUSSION.

The weights of total fatty acids (combined in various forms and free) average about 13 per cent higher in the anemic animals than in the normals, and the total unsaponifiable matter (mainly cholesterol) about 8 per cent higher in the anemics. The iodine values of the fatty acids average about 13 per cent higher in the anemic animals. The high weights for fatty acid in the anemic plasma occur only in the lecithin and in the first two ester fractions; in the other fractions they are below the normal weights. The high weight for unsaponifiable occurs only in the first ester fraction (33 per cent), in the others it is either about the same or below that for normal. The iodine values of the fatty acids are higher throughout the fractions in the anemic animals, with the single exception of the free fatty acid. The differences in iodine values between anemic and normal animals are greatest in the lecithin (23 points) and in the third (17 points) and fourth (29 points) fractions.

All of the ester fractions in both normal and anemic plasma yield more fatty acid than corresponds to any known ester of cholesterol and they, therefore, probably all contain ordinary fat (triglycerides).

The nature of the fatty acid mixture combined in the ester fractions has yet to be determined. In an earlier series of examinations¹ it was found that the solid fatty acid (mainly palmitic) constituted about 25 per cent of the total fatty acid mixture in both normal and anemic dog plasma. The amount of unsaturated acid precipitable by bromine in cold ether (three and four double bond acids) has not been found to be over 10 per cent in the few samples examined. The remaining unsaturated acids may be considered as oleic and linolic since the work of Hürthle (13) indicates that the acids, other than palmitic, are probably C_{18} acids. The average iodine number of the fatty acids in the ester fractions of anemic dogs is 130. Correcting for 25 per cent of palmitic acid which absorbs no iodine, the iodine number of the unsaturated acids would be 173. The iodine value of oleic acid is in round numbers 90, of linolic acid, 180. Neglecting the small amounts of more unsaturated acids a calculation shows that in such a

¹ Unpublished data.

mixture linolic acid is present to the extent of about 90 per cent and oleic to 10 per cent. In normal dogs the average iodine value of the ester fraction is 113 which would correspond to the 68 per cent linolic and 32 per cent oleic acid. Cholesterol combining with palmitic acid would require 66 per cent of its weight of fatty acid; with oleic or linolic about 73 per cent; for the mixture of the two as above, 71 per cent. A maximum then of about 71 per cent of the weight of cholesterol found might be present in combination as cholesterol ester. On this basis Fraction I of the anemic series would contain 31 per cent excess fatty acid; Fraction II, 36 per cent; Fraction III, 56 per cent; and Fraction IV, 74 per cent. On the same basis (combined fatty acid equals 71 per cent of the weight of unsaponifiable matter) the excess of fatty acids in the ester fractions of normal plasma would be: Fraction I, 26 per cent; Fraction III, 41 per cent; and Fraction IV, 70 per cent. As noted above these values probably represent largely fat and are almost certainly minimal, since there is an unknown but considerable amount of free cholesterol present which has been calculated as combined. The rise in percentage of excess fatty acid over that necessary for combination with the cholesterol in the later fractions indicates that fat tends to accumulate in the later fractions. The relatively high iodine values of the fatty acids of these fractions (129 to 131) in the anemic series over those in the normal (102 to 106) indicate that the excess fat in the anemic plasma is more highly unsaturated than that in the normal, and both sets of values show that the fat of the blood is not the unchanged fat of the depots, which has an iodine value of about 70, but represents rather a fat which, like that of the liver, has undergone considerable desaturation.

The greatest differences in iodine-combining power between anemic and normal dogs are to be found in the lecithin fraction and Fractions III and IV; therefore, in the lecithin and fat, indicating that these substances are most strongly affected by the conditions due to the anemia.

Lecithin Fraction.—The lecithin fatty acid averages 33 per cent higher in the anemic animals than in the normals. Owing to decomposition during preparation, the lecithin found probably does not represent all that was present in the original plasma, but even if it be assumed that all the free fatty acid originates from

decomposed lecithin, which is not likely, the sum of lecithin fatty acid plus free fatty acid still averages 30 per cent higher in the anemic plasma. The iodine values of the fatty acids of the lecithin fraction average 24 per cent higher in the anemic plasma than in the normal. The fatty acids of the free fatty acid fraction average only slightly higher in the anemic plasma than in the normal, but the sum of the two groups of fatty acid still gives a substantially higher value for the iodine number in the anemic plasma. The data submitted, then, leave no room for doubt that the content of lecithin is considerably greater in anemic plasma than in normal plasma, and also that the fatty acid constituents are considerably more unsaturated. The lecithin fatty acids in percentage of total fatty acids are not notably greater in the anemic plasma than in normal plasma (26.5 for anemic, 23.3 for normal), so that the lecithin is not out of balance with the remaining combined fatty acid:

The iodine values of the fatty acids of the lecithin fraction in both series are about the same whether the amount of lecithin in the plasma be high or low. Thus in the series of anemic animals the six highest weights (average 1.4 gm. per 1,000 cc.) have an average iodine value of 123 and the six lowest (average 0.362 gm. per 1,000 cc.) have the same average iodine value. Similarly, in the series of normals, the six highest weights (average 1.035 gm. per 1,000 cc.) have an iodine value of 102, while the six lowest (average 0.172) have an average iodine value of 97. If the very considerable variations in concentration of lecithin are to be laid to decomposition during extraction and preparation, which is probable, rather than to normal variation, the decomposition takes place equally with respect to the saturated and unsaturated acids, and not, as Levene and coworkers (14) have found for the action of cobra venom, with the liberation of the unsaturated acid and formation of lysolecithins. Moreover, in the few cases where it has been possible to collect enough anemic plasma lecithin for analysis (see below), the percentage of fatty acid obtained (64 to 67 per cent of the weight of the lecithin) indicated that the lecithin was intact.

Analyses.—A sample of anemic dog plasma lecithin prepared by three separations from ether by acetone gave the following data. Weight of sample 6.3 gm. which, corrected for unsaponifiable

residue of 0.37 gm., gave 5.93 gm. It yielded 4.00 gm. of fatty acid or 67.4 per cent. 3.7 gm. of the fatty acid by Twitchell's method (15) gave 1.85 gm. of liquid acid with an iodine number of 207, and 0.54 gm. of solid acid with iodine value of 14 and melting point of 55–61°C. The difference between the sum of the solid plus the liquid acid and the weight of the fatty acids originally taken was in part made up of a sticky lead compound, insoluble in boiling alcohol and partly soluble in ether. The ether-soluble portion yielded 0.45 gm. of a waxy material with an iodine number of 63. The portion insoluble in ether yielded 0.22 gm. of solid with an iodine value of 25. Judging by the iodine value, the liquid acids in this sample consisted mainly of linolic acid with some admixture of a more highly unsaturated acid. The solid acids consisted mainly of palmitic acid. The gummy material was possibly oxidation products of the unsaturated acids.

A second sample of anemic plasma lecithin gave data as follows: 4.36 gm. of lecithin, corrected for 0.284 gm. of unsaponifiable matter, yielded 2.63 gm. of fatty acid; i.e., 64.5 per cent. The fatty acid mixture on separation by Twitchell's process gave liquid acid 1.33 gm., iodine number 150; solid acid 0.46 gm., ether-soluble residue 0.112 gm., iodine number 87 (Pb salts insoluble in boiling alcohol but soluble in ether). The proportion of solid to liquid acids in this sample was about 1:3. 1 gm. of liquid acids was dissolved in cold ether and treated with excess bromine to form the bromine addition products. The white precipitate was washed twice with ether, dried, and weighed, giving a weight of 0.43 gm. The product was boiled with benzene to separate the triple bond derivatives, the solution cooled and let stand for 24 hours, after which the benzene was decanted, the residue washed with ether and again dried and weighed. The insoluble product weighed 0.323 gm. The benzene solution and ether washings were evaporated, dried, and weighed, yielding 0.100 gm. Calculating these values to terms of free C_{18} acids, there is obtained 0.096 gm. of tetra-unsaturated acid, 0.036 gm. of tri-unsaturated acid, a total of 0.132 gm. of acids more unsaturated than linolic, constituting 13 per cent of the liquid acids or about 9 per cent of the total acid.

The analytical data show that these two samples of the lecithin fraction in anemic plasma contained 64 to 67 per cent of fatty acid, consisting of about 1 part of solid acid to 3 parts of liquid acid.

The liquid acids consisted of a small proportion of a fatty acid with four double bonds, less of an acid with three double bonds, and mainly of a mixture of an acid of two double bonds, presumably linolic, and one with one double bond, probably oleic.

Ester Fractions.—As separated in this work these fractions in both normal and anemic plasma are mixtures of the cholesterol esters of saturated and unsaturated acids together with fat and probably small amounts of compounds of unknown nature. Considered together the saturated acids, mainly palmitic acid, constitute about one-fourth of the total combined acid, the unsaturated acids about three-fourths. The unsaturated acids appear to consist mainly of members of the linolic series ($C_nH_{2n-4}O_2$), and probably largely of linolic acid itself. There is a small amount of the more highly unsaturated acids—three and four double bonds—and a varying amount of oleic acid. The main difference between normal and anemic plasma appears to be that the unsaturated acids in the esters of anemic plasma contain a larger percentage of linolic acid and a correspondingly smaller percentage of oleic acid than in normal plasma.

As separated, the first fraction is generally well crystallized, the second fraction separates as an oil, and the third as shining flat plates which are so soft that shaking or stirring the liquid causes them to fuse together. The second fraction in both normal and anemic animals is characterized by having fatty acids with the highest iodine value of all, and by the fact that this iodine value is practically the same for both anemic and normal plasma.

Analyses of combined samples have been made as follows: A sample of Fraction I, recrystallized several times from methyl alcohol, yielded a well crystallized white product which on analysis gave the following data. Unsaponifiable 0.212; fatty acid 0.134; (theory for cholesterol palmitate 0.139); iodine number of fatty acid 45. This fraction thus contained a large amount of cholesterol palmitate, although judging by the iodine value as separated, it ordinarily had also a notable proportion of the esters of the unsaturated acids. The unsaponifiable matter in both this and the following sample was nearly white in color, well crystallized, and evidently nearly pure white cholesterol.

A collected sample of Fraction II from anemic dog plasma was thrice recrystallized according to Hürthle's method (13). A color-

less oil was obtained which on saponification and analysis gave the following data. Unsaponifiable matter 0.988; fatty acids 0.717; (theory for cholesterol linolate 0.719); iodine number of fatty acids 183. Of the fatty acids 0.704 gm. of liquid acid was obtained, indicating that the fatty acid was practically all liquid acid. The iodine number of this acid was, however, relatively low (143), indicating that in spite of the precautions taken to prevent it, considerable oxidation had taken place during the separation. The iodine number obtained for the original acid mixture (163) bears out the belief that this fraction consisted mainly of cholesterol linolate.

Free Fatty Acids.—The free fatty acid fraction may be considered in connection with the lecithin fraction, since there is considerable similarity in the nature of the fatty acids in both and a strong possibility that the free fatty acids originate to a considerable extent in lecithin decomposed during the separation.

The amounts of free fatty acid are considerable, averaging 0.789 gm. per 1,000 cc. in the anemic plasma and 0.410 gm. per 1,000 cc. in normal plasma, as compared with 0.569 and 0.627 gm. for the corresponding values of lecithin fatty acids.

No relation can be noted between the amount of lecithin and the amount of free fatty acid in any given sample, although the highest values for free fatty acids were obtained in two samples, 3.29 and 3.30, where the lecithin obtained was too small for analysis. The greater the amount of free fatty acid the higher the iodine number. Thus in the anemic animals, the ten highest (average 0.94 gm. per 1,000 cc.) have an average iodine value of 127, the ten lowest (average 0.21 gm.) an iodine value of 121. In the normals, the six highest (0.65 gm.) have an iodine value of 129, the six lowest (0.246 gm.) an iodine value of 107. The absolute amount of free fatty acid is about 20 per cent greater in the anemic plasma (average 0.506 gm.) than in the normal (0.410 gm.), but the iodine absorption values are practically the same in both. As to the mode of occurrence of the free fatty acids, the evidence at present available (16) indicates that they do not exist free in the living plasma. Soluble soaps would appear to be barred by the fact that these compounds of unsaturated acids injected into the blood stream produce hemolysis. (An acute experiment of this kind is of questionable value, since it gives little time for

adjustment.) Other forms of combination which might yield free fatty acid during the treatment outlined above would be: first, lecithin, in support of which is the marked similarity in composition between the free fatty acids and those in combination in the lecithin fraction; second, combinations with proteins, regarding which nothing is known; and third, loose combinations with cholesterol other than the cholesterol esters, the occurrence of which has been noted by Windaus, but regarding which also nothing is known.

Fat.—As noted above, there is a considerable proportion of the fatty acids in the ester fractions, which cannot be accounted for as cholesterol esters even if all the cholesterol is calculated as combined as ester, which is known not to be the case. The excess of fatty acid is much higher in the third and fourth fractions, from which the free fatty acid has been removed, than in the first and second fractions which may contain it. Other known ester combinations of the fatty acids, such as sphingomyelin and the cerebrosides, are probably excluded because of their insolubility in alcohol and acetone. There remains only the glycerol esters to be considered and since the triglycerides (fat) are known to be present in blood it is reasonable to assume that the excess fatty acid, which cannot be otherwise accounted for, is present as fat. As to the form in which the fat is present, the fasting plasma as used in the present work was almost always clear by transmitted light, but gave a marked Tyndall effect and a considerable cloudiness when examined in the nephelometer. A small amount of fat was present in the form of particles, visible with the highest powers of the microscope—the chylomicrons or hemaconia—but the amount of material of this nature was too small to be of importance. The remaining fat and probably also the other insoluble lipid material, such as cholesterol and cholesterol esters, can be best accounted for as being in the colloidal conditions, the lecithins and plasma proteins acting as protective colloids. As to the nature of the fat, the iodine values of the fatty acid of Ester Fractions III and IV, which contain most of the fat, are lower than that of the fatty acids of Fraction II, but higher than that of the other fractions including the lecithin and free fatty acid fractions, so that, allowing for a considerable contamination from Fraction II, the fatty acids of the fat are probably not any less unsaturated than

those of the lecithin, and at any rate would be more unsaturated than the fat of the stores.

The lipoids of plasma of experimentally anemic dogs have thus been found to differ from those of normal animals in the following respects: The total fatty acid averages 13 per cent higher, the unsaponifiable matter 8 per cent, and the iodine values of the fatty acids 15 per cent higher in the anemic than in the normal plasma. The high weights for fatty acids occur only in the lecithin and first two ester fractions; in the others the weights are below the normals. The high weight for unsaponifiable occurs only in the first ester fraction (33 per cent). The iodine values are higher throughout all the fractions of anemic plasma, but the difference is greatest in the lecithin fraction and in the last two ester fractions which contain most of the fat, indicating that these two groups are most affected by the conditions produced by the anemia. Wide variations in the iodine value of the fatty acids are present in both normal and anemic samples. These are undoubtedly due in part to oxidation during separation and examination, but probably are mainly to be regarded as the result of varying amounts of unchanged tissue or food fat of relatively low iodine value.

The lecithin fraction is notably greater (30 per cent) in the anemic plasma than in the normal, and the constituent fatty acids are more unsaturated (iodine numbers average 24 per cent higher), indicating that the increase is in the lecithins of the unsaturated fatty acids.

SUMMARY.

The outstanding feature of the lipoids of anemic as compared with normal plasma was found to be the increased percentage and degree of unsaturation of the fatty acids, which is in general agreement with the somewhat irregular results in the literature regarding the blood lipoids in anemia. The differences are most marked in the lecithin fraction and in the fat. The unsaponifiable matter is increased, but to a less extent than the fatty acids. The significance of the increase of unsaturated acids is difficult to determine. The blood corpuscles have been found (17) to take part in certain phases of fat metabolism, and it may be that the diminished number of corpuscles in anemia is the cause of the phenomenon. On the other hand, the blood-forming organs, such as the marrow

of the long bones, are undoubtedly stimulated by the bleeding, and the suggestion that has been offered for the great increase in amount and degree of unsaturation of the blood lipoids, which is observed after repeated hemorrhages in rabbits, may apply here; *i.e.*, the forcing out of the fat marrow by the blood-forming red marrow (18).

One additional point may be emphasized as significant—the increase of unsaturated acids is the *result* and not the *cause* of the anemia. The experimental animals were normal in every respect except that they had been deprived of part of their blood which they were replacing in a normal manner. The belief that the presence of abnormal amounts of unsaturated acids is a cause of anemia thus loses some of its support.

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URIC ACID DETERMINATIONS IN BLOOD.

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A review of the literature on uric acid covering the past 12 years must astonish the reader rather in the consistency of the findings than in the discrepancies revealed by the use of many different reagents and methods. It was pointed out by Folin (8) in 1922 that there were five different uric acid reagents then in use for the one specific purpose of estimating uric acid in blood or urine. Differences in method, however, were not confined to differences in uric acid reagents, for even by 1922 the original Folin and Denis method (11) had been modified in many other ways. It is not our purpose to deal with the many methods and modifications of methods for the estimation of uric acid but to refer only to those immediately affecting the subject matter of this paper. The findings reported here have been arrived at by the use of a "direct" and an "indirect" method on the same bloods.¹ Our technique for the two methods will be detailed later.

The first so called "direct" method for the estimation of uric acid was introduced by Benedict (2). Some form of direct method now appears to have been generally adopted, at any rate in most hospitals and clinical laboratories. Little evidence against its reliability has been adduced from any quarter.

¹ The terms "direct" and "indirect" used throughout this paper, though not strictly accurate, are the best we can suggest. Any method in which the colour is developed from the Folin and Wu filtrate without further treatment we term a direct method; and any method which involves the intermediate precipitation of uric acid from this filtrate we term an indirect method.

It is unfortunate that no method for the estimation of uric acid in blood has been proved to be perfectly reliable. It is accepted that the silver lactate solution which apparently precipitates uric acid from blood will not precipitate uric acid from an aqueous solution of corresponding strength. The uric acid is presumably adsorbed by some other silver salts of the uric acid fraction. The mechanism of the precipitation is so far unexplained, so it is conceivable that this factor or set of factors responsible for the complete precipitation of uric acid in one blood may not operate in the case of another blood. The evidence from results suggests that complete precipitation takes place in all cases, but this is at best an empirical conclusion.

It is, on the other hand, admitted that the direct method measures accurately the uric acid content of aqueous or simple solutions. It gives values in human blood which agree very well with those given by the indirect method in a large proportion of cases, but in many instances the divergence is beyond the range of experimental error. We think that this divergence has not been sufficiently emphasized. There are, further, indications in the literature that difficulties are encountered when the direct method is used on animal bloods. On attempting to measure uric acid added to sheep's blood Folin (8) found too high results by the direct method of Benedict, suggesting an interfering substance which he thought might possibly be found in human blood also. Benedict (3) objected to Folin's criticism of his method on this ground and thought that uric acid determination was a "different problem for the blood of each species." This is probably overestimating the difficulties. Benedict states that his method is designed for human blood only.

It is true that Folin's finding is no disproof of the reliability of the direct method on human blood and he has himself tentatively offered a direct method (8). Yet Folin's finding is somewhat disturbing, for, as far as we know, there is no qualitative distinction between human blood and the blood of animals such as the ox, dog, cat, or rabbit. The outstanding difference, until recent date, between human blood and the blood of such animals lay in uric acid. Recent investigation, however, seems to show that this distinction has broken down, as animal bloods contain uric acid if only in very small amounts (10). And so, if animal bloods do contain a substance which interferes with the estimation of uric acid by the direct method, it appears probable that this substance will occasionally be met with in human bloods. Our results indicate that this is indeed so.

Our findings appear to elucidate some obscurities in the recent literature on uric acid. An understanding of their significance necessitates a brief review of the literature in regard to: (a) the uric acid content of normal human blood, (b) the distribution of the uric acid of whole blood in corpuscles and plasma, and (c) the values for uric acid in certain animal bloods.

(a) Normal Human Blood.

1. *Average Normal Values by Direct and Indirect Methods.*—An average figure for uric acid in normal human blood may be obtained from the results

published by Folin and Denis (12). A consideration of Tables II and V of their paper yields from 49 unselected cases an average of 1.84 mg. of uric acid per 100 gm. of blood. From the paper by Folin and Wu (14) we obtain a more reliable figure after the introduction of tungstic acid as a protein precipitant and the use of silver lactate in lactic acid in place of silver magnesia mixture. In Table IV of Folin and Wu's paper the analyses of 40 bloods are presented. The first 37 of these may be regarded as normal and give an average of 2.43 mg. of uric acid per 100 cc. of blood.

Turning now to the first published analyses with the direct method of Benedict (2) we find in Table I the uric acid values in 50 different bloods. In 10 of these cases the non-protein nitrogen is over 55 mg. per 100 cc. and these will, for our purpose, be discarded. The average figure for the remaining 40 cases is 3.43 mg. per 100 cc. This average is rather more than 40 per cent greater than that obtained from Folin and Wu's figures. Alongside the figures obtained by the direct method, Benedict shows the corresponding results obtained by the method of Folin and Wu and finds for the same 40 cases an average of 3.02 mg. which is approximately 0.4 mg. lower than that obtained by the Benedict method. The opinion of various workers who have tried both methods appears to be that the direct method gives on the average a slightly higher result than the Folin and Wu method. This average difference is small and has previously been attributed to loss by the extra manipulation in the Folin and Wu procedure; but we would explain it in another way.

2. *Disagreement between Direct and Indirect Methods on Whole Blood.*—The calculation of averages in the foregoing fashion tends to obscure a more important matter; that is, the large discrepancies between the values obtained by the direct and indirect methods in individual bloods. Comparing the results obtained by the direct method of Benedict with those obtained by the indirect method of Folin and Wu, we find in Benedict's paper that of the 40 cases above referred to 30 show a greater uric acid by the direct method, 9 a lower value, and in 1 case the two methods agree exactly. Of the 30 there are 12 in which the direct value exceeds the indirect value by 0.5 mg. or less, and 18 by more than 0.5 mg. In 8 of the cases the direct value exceeds the indirect value by 1.0 mg. and over, the greatest difference being 1.7 mg.

Discarding for the present the question as to whether a difference of 0.5 mg. per 100 cc. can be attributed to experimental error, it can hardly be denied that differences of 1.0 mg. and over call for some explanation. We shall show instances of even greater discrepancies in the values by the two methods.

(b) *Distribution between Corpuscles and Plasma of Human Blood.*

Rose (17) points out that it is difficult to harmonize the observations on the distribution of uric acid between corpuscles and plasma. Out of 20 bloods Bornstein and Griesbach (5) found 2 with equal distribution, 13 in

which the uric acid was greater in the corpuscles, and 5 in which it was greater in the plasma. Of 104 cases Theis and Benedict (18) found 51 with equal distribution, 8 with greater concentration in the cells, and 45 with greater amounts in the plasma. In analyses of 20 normal bloods Wu (19) uniformly found about twice as much in the plasma as in the cells, and this was so even in pathological conditions. Plass (16) in 1922 showed that uric acid was generally in greater concentration in the plasma though under certain conditions it was greater in the corpuscles.

It should be noted that Bornstein and Griesbach and Theis and Benedict employed colloidal iron for protein precipitation and the method of Folin and Denis (11) for the estimation of uric acid. Wu and Plass employed the tungstic acid filtrate and used silver lactate in lactic acid instead of silver magnesia mixture for the uric acid precipitation.

(c) *Animal Bloods.*

The first uric acid values obtained by modern methods on animal bloods are those published by Folin and Denis (12, 9). Here we find for the rabbit 0.05 mg. per 100 cc.; for the ox 0.2 mg.; and for the cat 0.2 mg. Brown and Raiziss (6) publish the only values we have seen in the literature obtained by a direct method on animal bloods. By the direct method of Benedict they found in 6 rabbits values ranging from 1.9 to 3.7 mg., calculated as uric acid per 100 cc. of blood. They also give values for the blood of 2 guinea pigs and 2 sheep. Folin, Berglund, and Derick (10) give a few values for the plasma of the rabbit and dog, using phosphotungstic acid directly.

There is thus evidence in the literature that rabbit blood may give a uric acid value by the direct method within the range of values found in human blood.

EXPERIMENTAL.

Our experimental work is presented in the reverse order to our treatment of the literature. We have indicated that a consideration of the method of estimation is important in attempting to draw conclusions from the literature on uric acid. For that reason we detail the procedure used throughout the following work.

The *direct* method is performed as described by Benedict (2). 5 cc. of the Folin and Wu filtrate are put in a test-tube with 5 cc. of water. To this are added 4 cc. of 5 per cent sodium cyanide (with 2 cc. of strong ammonia per liter) and 1 cc. of Benedict's arsenophosphotungstic acid reagent.

The *indirect* method is as follows: To 5 cc. of the Folin and Wu filtrate in a graduated centrifuge tube are added 5 cc. of 5 per cent silver lactate in 5 per cent lactic acid—made according to Benedict (2). This is centrifuged till the supernatant fluid is clear, the supernatant fluid is discarded, and 1 cc. of 10 per cent NaCl in 0.1 N HCl is added to the precipitate which is stirred with a fine glass rod to bring all of it into contact with the chloride

solution. The tube is shaken without tilting, made up to 10 cc. with water, and centrifuged. The supernatant fluid is poured into a clean test-tube and 1 minute allowed for drainage; then are added 4 cc. of the same sodium cyanide and 1 cc. of the same arsenophosphotungstic acid as used in the direct method.

The uric acid standard used is that recommended by Benedict and Hitchcock (4). Two concentrations of standard are used with each test. From a standard with 4.0 mg. of uric acid per liter, are taken 5 and 2.5 cc. in two test-tubes, and 5 and 7.5 cc. of water are added respectively. To both are added 4 cc. of sodium cyanide and 1 cc. of arsenophosphotungstic acid as above, at the same time as these reagents are added to the two test-tubes representing the solutions from the direct and indirect methods. Each of the four tubes is then inverted once and all are placed at the same time in a water bath just on the point of boiling. After 2 minutes they are all removed, cooled 3 minutes in the air, and read according to the more suitable standard in a modern Duboscq colorimeter.

Plasma is precipitated in the same way as whole blood. 2 volumes of sodium tungstate and of $\frac{2}{3}$ N sulfuric acid are used for the corpuscles with a 1 in 20 dilution; so that 10 cc. of filtrate are used in place of 5 cc. as used for whole blood and plasma.

The direct and indirect methods used are thus the same apart from the precipitation by silver in the indirect method.

An excess of silver lactate is used to ensure complete precipitation of the uric acid (Folin (8)).

Results on Animals by the Direct and Indirect Methods.

The results obtained by the two methods on a number of animal bloods are shown in Table I.

The colour is expressed as uric acid for convenience. The values obtained by the direct method for 12 rabbits, 4 dogs, 2 cats, 2 guinea pigs, and 4 oxen, are about the same as normal human uric acid values. The values by the indirect method are higher than they should be on account of the colour given by the reagents, and from the fact that these were obtained against a 2 mg. per liter uric acid standard. Had a 0.5 mg. per liter standard been used we should probably have found values of less than 0.2 mg., so that the apparent difference in the figures by the direct and indirect methods is less than it should be. Using water in place of blood filtrate, what may be called the "water value" of 0.68 mg. is obtained. The maximum divergence of five determinations against a 2 mg. per liter uric acid standard was 0.1 mg., so that the indirect values as a whole coincide with

this water value and indicate no uric acid. Ox 2 shows the highest value, and, as we shall show, this probably indicates some free uric acid.

It should be noted here that we are not concerned as to whether there is a trace of uric acid in these bloods or not. We should require to use a much weaker standard to ensure accuracy in the low readings from the indirect method, but we have not done

TABLE I.

Animal.	Uric acid per 100 cc. whole blood.			Animal.	Uric acid per 100 cc. whole blood.		
	Direct.	Indirect.*	Difference.		Direct.	Indirect.*	Difference.
	mg.	mg.	mg.		mg.	mg.	mg.
Rabbit.				Dog.			
1	3.19	0.67	2.52	1	1.63	0.53	1.10
2	2.11	0.58	1.53	2	1.96	0.50	1.46
3	1.66	0.69	0.97	3	2.61	0.55	2.06
4	2.83	0.56	2.27	4	1.90	0.41	1.49
5	3.54	0.52	3.02	Cat.			
6	1.82	0.70	1.12	1	2.50	0.75	1.75
7	2.24	0.70	1.54	2	1.54	0.49	1.05
8	2.57	0.69	1.88	Guinea Pig.			
9	3.75	0.58	3.17	1	2.39		
10	2.73	0.40	2.33	2	2.10	0.74	1.36
11	3.33	0.53	2.80	Ox.			
12	2.11	0.50	1.61	1	3.19	0.66	2.53
				2	3.33	0.94	2.39
				3	2.30	0.43	1.87
				4	2.51	0.51	2.00

* It is especially emphasized that the indirect values in Tables I and II and the indirect values for rabbit blood in Table III do not represent uric acid or substance X, but are mainly due to the blank values in the reagents as pointed out in the text.

so as we think this can better be settled with reagents which in themselves give no colour. These we have not yet been able to obtain (Folin and Trimble (13)).

The substance responsible for the colour by the direct method is presumably not uric acid. (We shall refer to it as substance X.) We think this is evidenced by considerations other than the fact that it is not precipitated by silver lactate. It is not dis-

tributed between corpuscles and plasma in the manner of distribution of uric acid. Table II makes this clear.

Table II presents the distributional analyses of the blood of a rabbit, and the bloods of Oxen 1 and 2. It should be noted that the values found in the whole blood by the direct method check well with those calculated from the corpuscular percentage. As has been pointed out the values obtained by the indirect method are not true values and no attempt need be made to balance them. The direct method shows that on an equal volume basis the corpuscles contain three to five times as much of substance X as the plasma.

TABLE II.

Animal.	Corpuscles.	Uric acid per 100 cc.				Plasma Corpuscles	Method.
		Corpuscles.	Plasma.	Whole blood.			
				Found.	Calculated.		
	vol. per cent	mg.	mg.	mg.	mg.		
Rabbit	42.9	5.00	1.60	3.06	3.05	0.32	Direct.
		0.63	0.51	0.76			Indirect.*
Ox 1.	48.4	5.88	1.26	3.19	3.49	0.21	Direct.
		0.65	0.67	0.66			Indirect.*
" 2.	44.0	5.36	2.16	3.33	3.55	0.40	Direct.
		0.80	1.06	0.94			Indirect.*

* See foot-note to Table I.

The value of 1.06 mg. in the plasma of the blood of Ox 2 is in keeping with the high value on the whole blood—indicating, probably, the presence of free uric acid in this ox blood.

Behaviour of Bloods on Standing.

It has been found that in bloods showing an initial large difference in the direct and indirect values, this difference diminishes if the blood is allowed to stand under toluene over a period of days at room temperature. The values obtained in Oxen 1 and 2 are interesting in this respect and are, by the way, confirmatory of the finding by Benedict and his coworkers of combined uric acid in ox blood (1, 7).

Uric Acid in Blood

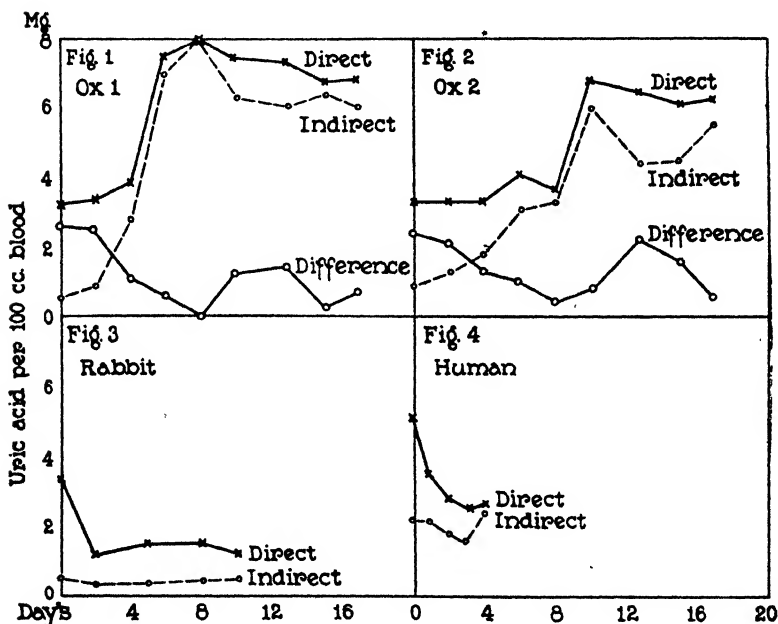
TABLE III.

Ox 1.				Ox 2.				Rabbit.				Human.			
Date.	Direct value. mg. per 100 cc.	Indirect value. mg. per 100 cc.	Difference. mg. per 100 cc.	Date.	Direct value. mg. per 100 cc.	Indirect value. mg. per 100 cc.	Difference. mg. per 100 cc.	Date.	Direct value. mg. per 100 cc.	Indirect value.* mg. per 100 cc.	Difference. mg. per 100 cc.	Date.	Direct value. mg. per 100 cc.	Indirect value. mg. per 100 cc.	Difference. mg. per 100 cc.
June 17	3.19	0.66	2.53	June 17	3.33	0.94	2.39	July 21	3.41	0.50	2.91	Aug. 5	5.08	2.17	2.91
" 19	3.22	0.89	2.33	" 19	3.30	1.22	2.08	" 23	1.13	0.37	0.76	" 6	3.61	2.14	1.47
" 21	3.84	2.78	1.06	" 21	3.15	1.81	1.34	" 26	1.47	0.35	1.12	" 7	2.85	1.90	0.95
" 23	7.53	6.99	0.54	" 23	4.02	3.00	1.02	" 29	1.43	0.40	1.03	" 8	2.45	1.64	0.81
" 25	8.10	8.10	0	" 25	3.52	3.17	0.35	Aug. 1	1.26	0.49	0.77	" 9	2.68	2.44	0.24
" 27	7.44	6.19	1.25	" 27	6.82	6.00	0.82								
" 30	7.32	6.00	1.32	" 30	6.52	4.28	2.24								
July 2	6.52	6.32	0.20	July 2	6.06	4.34	1.72								
" 4	6.60	6.00	0.66	" 4	6.25	5.55	0.70								

* See foot-note to Table I.

Table III shows the behaviour on standing of the blood of Oxen 1 and 2, of a rabbit and of a human with an initial large difference in the direct and indirect values.

In Ox 1 there is an initial difference of 2.53 mg. There is little change in the values during the next 2 days. 2 days later still the indirect value has risen and after 2 more days both values have more than doubled their original values. On June 25 both values are 8.10 mg. After this there is an irregular behaviour.



FIGS. 1 TO 4.

If the direct and indirect values are charted as in Figs. 1 to 4, it is clear that there are at least two processes at work.

After standing 4 days there is a rapid development of free uric acid borne out by both methods. But more important from our point of view, there is a continuous diminution in the difference by the two methods up to 8 days. By graphing this difference we get the rate of disappearance of substance X from the blood. Graphing a difference curve takes off the mask, as it were, superimposed by the development of free uric acid.

The values from Ox 2 taken from Table III and charted on Fig. 2 show the same behaviour, but in a less diagrammatic fashion.

Values obtained in the same way from the blood of a rabbit are given in Table III and charted in Fig. 3. The curves in this case are free from the masking effect due to combined uric acid, so that the direct value runs in a similar direction to the difference curve in Figs. 1 and 2. In other words, the direct value curve in the rabbit is the same curve as the difference curve in Figs. 1 and 2.

Human Blood.

The foregoing considerations have led us to a probable explanation of the problem of why a direct method generally gives a higher value for human blood than does an indirect method. A difference even of 0.5 mg. cannot in our experience be accounted for by the likelihood of loss by the extra manipulation inseparable from the indirect procedure. For in many cases the values by the two methods coincide to within 0.1 mg. and the precipitation can be repeated several times on the same filtrate by a competent worker with practically identical results. So far as our experience goes there is no demonstrable loss of uric acid by the silver lactate precipitation. This is in accordance with the findings of Brown and Raiziss (6). It is at least most unlikely that divergences of 1.5 to 2.5 mg. can be explained on the assumption of loss by technique or incomplete precipitation.

In the majority of human bloods it is, of course, admitted by all that the direct and indirect methods give values which agree very closely. Benedict's own findings in this regard (2) are in agreement with those of other workers. Greater divergences than any shown in the 50 patients investigated by Benedict are occasionally found and these are not explained in the literature up to date.

About the same time as the abnormal behaviour of animal blood towards the direct method was first noted, one of us (F. M. R. B.) was investigating some patients suffering from a skin affection known as "nickel rash" which is acquired by some workers in nickel refineries. The large differences between the results obtained by the direct and indirect methods was thought at first to be an accompaniment of the nickel rash, but the

difference still persisted after treatment and cure, and differences of like magnitude were found in six apparently healthy men who were working in the same factory.

Table IV presents the results obtained on fourteen men. Three of these were repeated at intervals of 2 weeks.

The remarkable feature of Table IV is the great differences in the values obtained by the direct and indirect methods. In

TABLE IV.

Case No.	Remarks.	Non-protein nitrogen.	Uric acid per 100 cc.		
			Direct method.	Indirect method.	Difference.
		<i>mg. per 100 cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	Nickel rash.	40.0	4.05	2.90	+1.15
2	" "	49.5	3.70	3.89	-0.19
3	" "	45.9	5.31	3.71	+1.60
	2 wks. later, after treatment.	32.6	4.22	1.87	+2.35
4	Nickel rash.	44.1	4.90	2.60	+2.30
	2 wks. later, after treatment.	40.9	4.50	2.30	+2.20
5	Treated patient.	49.0	2.59	1.85	+0.74
6	Cured patient.	43.2	3.45	3.11	+0.34
7	Nickel rash.	29.5	4.41	3.10	+1.31
	Treated.	45.0	3.40	1.90	+1.50
8	Nickel rash.	48.0	5.08	2.17	+2.81
9	Normal.	33.6	3.24	1.88	+1.36
10	"	36.9	5.00	2.76	+2.24
11	"	36.0	5.30	3.24	+2.06
12	"	45.5	4.28	2.81	+1.47
13	"	36.0	4.00	2.69	+1.31
14	"	37.5	4.00	2.30	+1.70

two cases, Nos. 3 and 8, the difference is greater than the indirect value. Values greater than 5.0 mg. would be interpreted by many clinicians as evidence of uric acid retention. That is, some of the values obtained by the direct method indicate a pathological condition, though the corresponding indirect values indicate a normal condition.

The blood of Patient 2 shows a lower value by the direct than by the indirect method. This is not uncommon and we have

encountered greater differences in this direction in other studies. Several examples are shown by Benedict (2) where there is no evidence of retention as proved by the non-protein nitrogen figures. This is important, for out of four cases with high non-protein nitrogen values three showed a greater value by the indirect than by the direct method. Benedict states in regard to this point that "theoretically the lower figures should be regarded as correct, and it can be shown that there is experimental evidence in favor of this view." We are unable to admit, however, that Benedict produces sufficient evidence to show that the low results by the direct method are more dependable than the higher results by the indirect method even when there is retention. For with average non-protein nitrogen values one must postulate something of the nature of a colour-inhibiting substance in such cases as show a higher indirect than direct value. (We shall provide further evidence on this point in the sequel.) And with high non-protein nitrogen values it would appear that there is more likelihood of the presence of interfering substances in the direct method than in the indirect—unspecific as the silver lactate precipitation may be.

It is hardly necessary to state that we were at first sceptical about our results in Table IV, and they were checked by every means we could think of. One of us (B. A. E.) had been estimating uric acid to the extent of about twenty bloods per week for 6 months previously on hospital patients—mainly by the direct method. These had been checked from time to time by the indirect method with only occasional discrepancies. The results of Table IV were obtained with the same reagents as those used in hospital bloods which showed no marked differences. On several occasions the blood was checked directly against a hospital blood done at the same time. Thus along with the blood of No. 8 which shows a direct value of 5.08 mg. and an indirect value of 2.17 mg. another blood was taken which showed corresponding values of 5.45 and 5.04 mg.

The behaviour of the blood of No. 8 on standing is shown in Table III and charted in Fig. 4. Here we find a pronounced drop in the direct value which reaches a minimum at the end of 3 days. The similarity of its behaviour to that of the rabbit blood in Fig. 3 is remarkable.

The changes in the uric acid content of human blood after a few hours incubation, recorded by Bornstein and Griesbach, are again puzzling. Had those authors used a direct method of estimation we should have concurred in their findings, and attributed some of these to the decomposition of this unknown substance.

It did not occur to us until the results of Table IV had been completed that a distributional analysis of some of those bloods might throw more light on the subject. Since then we have had more difficulty in getting bloods of this type. We have, however, succeeded in getting two with differences greater than 1.5 mg. and several others with a difference of more than 0.5 mg. The distributional analyses of these are enlightening. They are presented in Table V along with a number of normal bloods.

The first seven cases all show differences of more than 0.5 mg. per 100 cc. of whole blood. The values for the whole blood, calculated from the values found for the corpuscles and plasma with the help of the corpuscle percentage, agree well with the values actually found in the whole blood by both direct and indirect methods. The calculated direct value in Case 1 is an exception to this, probably owing to an error either in the whole blood or corpuscle figure. In Case 7 also the calculated values are a little higher than the values found, probably because too low a standard was used for the plasma readings. It will be seen that any difference found in the whole blood is intensified by the corpuscle figures. With a marked difference in the whole blood figures there tends also to be an increase in the plasma figure by the direct method. But in general the plasma figures by the direct and indirect methods are in agreement. The reality of large initial differences in the whole blood is thus borne out by the values found for corpuscles and plasma.

The other bloods in which there is a relatively low initial difference have proved more interesting than we anticipated. The blood of No. 8 is the first of this type which forced itself on our attention. This was from a normal man and the initial difference found between the two methods on the whole blood was -0.08 mg. which is well within experimental error. When we look at the corpuscle value, however, we find the direct method registers 1.02 mg. more than the indirect; the plasma figures

TABLE V.

Subject.	Cor- puscles.	Uric acid per 100 cc.				Plasma Corpuscles.	Method.
		Cor- puscles.	Plasma.	Whole blood.			
				Found.	Calcu- lated.		
	<i>vol. per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>		
1	45.4	6.66	4.72	6.45	5.69	0.71	Direct.
		3.04	4.51	3.35	3.80	1.48	Indirect.
2	43.2	4.69	3.61	4.11	4.18	0.77	Direct.
		1.95	3.05	2.40	2.57	1.56	Indirect.
3	35.3	4.61	2.60	3.16	3.31	0.56	Direct.
		1.40	2.17	Lost.	1.89	1.55	Indirect.
4	47.5	3.24	4.44	4.00	3.87	1.37	Direct.
		1.82	4.29	3.24	3.10	2.36	Indirect.
5	27.3	4.76	7.21	6.32	6.54	1.52	Direct.
		3.21	7.10	5.71	6.03	2.21	Indirect.
6	42.5	3.98	4.14	3.87	4.07	1.04	Direct.
		2.64	3.93	3.33	3.38	1.49	Indirect.
7	47.0	7.50	11.32	9.09	9.50	1.51	Direct.
		6.31	11.32	8.57	8.95	1.79	Indirect.
8	47.2	3.16	4.80	3.43	4.02	1.52	Direct.
		2.14	4.88	3.51	3.58	2.19	Indirect.
9	52.0	2.65	5.62	3.53	4.08	2.12	Direct.
		2.00	5.45	3.53	3.65	2.72	Indirect.
10	50.0	2.61	3.63	2.67	3.11	1.39	Direct.
		1.57	3.63	2.61	2.60	2.31	Indirect.
11	52.2	2.27	3.28	2.31	2.75	1.44	Direct.
		1.66	3.14	2.10	2.32	1.89	Indirect.
12	55.8	2.34	3.28	2.31	2.76	1.40	Direct.
		1.76	3.22	2.26	2.40	1.83	Indirect.
13	51.4	3.89	5.04	3.98	4.55	1.29	Direct.
		2.94	5.04	3.70	3.95	1.72	Indirect.
14	41.6	3.64	5.22	4.00	4.55	1.43	Direct.
		2.70	5.36	4.14	4.25	1.99	Indirect.

practically agree. The calculated figure for the whole blood is about 0.5 mg. higher than we actually find, although the indirect calculated values agree almost perfectly. We suspected an error here and the direct values were all repeated and found to agree to within 0.1 mg. of the results first found. To make still more certain we repeated both methods starting with the whole blood and again found substantially the same values for the three fractions.

Thinking that possibly the dilution of the corpuscles to 1 in 20 instead of 1 in 10 might affect the precipitation of uric acid by the silver lactate we repeated the determination of the corpuscle fraction of the blood of No. 9 with a dilution of 1 in 10. Our figures instead of 2.65 and 2.00 mg. were 2.50 and 1.86 mg. respectively. Approximately the same difference was maintained, although the figures were both slightly lowered, as would be expected.

Table V shows that the values for whole blood, found and calculated, agree better than one might expect. Taking into consideration that the corpuscle value is a little higher on account of the 1 in 20 dilution it might be said that the indirect values check almost perfectly, and this despite the extra manipulation and possibilities of loss which have always been evidenced against the reliability of the indirect method of Folin and his coworkers. On the other hand, the direct values found on the whole blood, especially in the more normal cases, do not check satisfactorily with the values calculated from the corpuscles and plasma, and this despite the fact that the procedure is so simple that there is hardly any possibility of error. It thus appears certain that there is a substance other than uric acid which produces a colour with arsenophosphotungstic acid, present in some human bloods to a marked degree and to a lesser extent in all human blood. This substance is for the most part in the corpuscles, but there are indications, especially when it is present in blood in larger amounts, that the plasma contains a small proportion.

DISCUSSION.

The contents of Table V are significant from several aspects. They provide indications of the presence of a hitherto unrecog-

nised substance in human blood. This substance is characterised by the facts that: (a) it is not precipitated along with uric acid from protein-free blood filtrates by silver lactate, (b) it is for the most part found in the corpuscles of human blood, and (c) it practically disappears from blood on standing. It is, further, not "combined uric acid."

This unknown substance in human blood is similar in the above respects to a substance in animal blood which gives a blue colour with the arsenophosphotungstic acid reagent. There is thus some evidence for the identity of the substance in animal blood with the substance in human blood.

We have insufficient data even to speculate on the nature of this new substance. It appears to be generally believed that polyphenols are responsible for some of the divergences in the methods. But it seems improbable that polyphenols—if we take resorcinol as a type—are responsible for the high values by the direct method. Benedict (2) showed that his reagent was scarcely affected by polyphenols. With five times as much resorcinol as uric acid in solution he obtained only a 15 per cent increase of colour; with 50 times as much, a 66 per cent increase. In two of the cases of Table IV is shown an increase of colour of more than 100 per cent, which cannot be accounted for by polyphenols since these occur in human blood to the extent of only 2 to 3 mg. per 100 cc. (15). The distribution of phenols between corpuscles and plasma might throw more light on this question, but we have been unable to find any such figures.

We think the results in Table V satisfactorily explain some hitherto unaccounted for discrepancies in the direct and indirect methods. When the direct value exceeds the indirect value by more than 0.2 mg.—a wide enough range for experimental error—the direct method is measuring a substance other than uric acid. And if in such a case the corpuscles are analysed, the divergence in the values by the two methods is even greater. We are thus forced to conclude that the average figure given by the Benedict direct method and shown at the beginning of this paper to exceed that of the indirect method of Folin and Wu by about 0.5 mg. of uric acid per 100 cc. of blood is too high a figure on account of the presence of another substance in human blood which augments the direct value.

It is also clear from Table V that the indirect value may be less than the direct value *on whole blood*, though we are unable to offer a satisfactory explanation of why this occurs.

With regard to the inconsistencies in the figures published on the distribution of uric acid in corpuscles and plasma, we think it significant that the quotient of the ratio of uric acid in plasma to uric acid in corpuscles gives by the direct method values quite parallel with those obtained by Bornstein and Griesbach and by Theis and Benedict. The plasma, according to our direct method and according to these workers, who used an indirect method, may have more or less uric acid than the corpuscles. Now, apart from the difference in the methods of protein precipitation, these workers used silver magnesia mixture to precipitate the uric acid; that is, the precipitation was made in alkaline and not in acid solution as used by Wu and by us. We thus suspect that at least some of substance X is precipitated by the old alkaline uric acid precipitant, on which assumption the discrepancies in the distributional figures are readily explained. Our distributional figures by the indirect method agree well with those found by Wu. The ratio of plasma to corpuscles in Wu's twenty cases gives the quotient 2.04. Our ratio for fourteen cases gives the quotient 1.93. In every case the plasma contained more uric acid than the corpuscles on an equal volume basis.

The distribution of uric acid in plasma and corpuscles in human blood is shown by different methods of estimation to vary for the same blood. The indirect method shows that the plasma contains on an equal volume basis about twice as much uric acid as the corpuscles. This we take to be the true distribution of uric acid between plasma and corpuscles. The direct method, on the other hand, almost invariably indicates a lower plasma:corpuscle quotient than does the indirect method. And this we explain as due to a substance other than uric acid mainly located in the corpuscles and augmenting the direct value obtained therefrom.

It is indicated in Table V that even in instances where the values obtained from whole blood by the direct method coincide with those by the indirect method it is accidental that the direct method is measuring the actual amount of uric acid in such cases.

Sometimes the values calculated and those found by the direct method do agree well, but in most cases the calculated value is greater and probably a more accurate direct value for whole blood than that actually found. It is difficult to frame even a tentative explanation for our findings in this regard. The direct method on whole blood gives a lower result than it should. If there be a colour-inhibiting substance in the whole blood why is it not also in the corpuscles or plasma or both?

It should be noted that the phosphotungstic acid reagent of Folin is no more specific for uric acid than is the arsenophosphotungstic acid of Benedict. The protein-free filtrate from the blood of a rabbit gave with arsenophosphotungstic acid 2.5 mg. as contrasted with 2.3 mg. per 100 cc. with phosphotungstic acid.

CONCLUSION.

The foregoing evidence leads us to conclude that there is a substance other than uric acid present in the blood of the rabbit, dog, cat, guinea pig, and ox, which interferes with any direct method for the estimation of uric acid in such bloods; the same or a like substance is present in variable amounts in human blood, and the presence of such a substance renders unreliable the values for uric acid obtained by any direct method of estimation with the reagents now at our disposal for that purpose.

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THE EFFECT OF HIGH FAT DIETS ON THE CONTENT OF URIC ACID IN BLOOD.*

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The present paper is a description of a phenomenon in the biochemistry of uric acid which we believe to be new. The nearest approach in the literature is an account by Lennox (1) of some observations on the rise in blood uric acid during the fasting treatment of epileptics. Our observation is that high fat diets producing a ketosis invariably raise the uric acid content of blood, and that often the rise in uric acid is accompanied by an increase in the non-protein N which is not attributable to an augmentation of the blood urea.

The subjects were normal women in various stages of pregnancy, except T-l-n., who was 14 days post partum and not nursing. Mainly two diets were used, labelled high fat diets Nos. 1 and 2, the details of which are given in Table I. No particular order was adopted in the giving of these diets, though diet No. 2 was used rather more frequently as it proved to be more popular. The diets were not adjusted to the calory needs of the subject, but were generally too low. The carbohydrate or protein diets mentioned in this paper are those detailed in a previous communication in this Journal (2). During the experimental period the subjects

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remained in bed under the supervision of a special nurse. Urine collections were made daily. Blood specimens were taken while fasting, and determinations were made on whole blood, using oxalate as anticoagulant.

The analytical methods used were the following.

Non-protein N.....	Folin and Wu (3).
Urea.....	Van Slyke and Cullen (4).
Uric acid.....	Benedict (5).
Chlorides.....	Whitehorn (6).
Total acetone.....	Van Slyke (7).
Serum protein....	Refractometer, using the conversion tables of Reiss (8).

In Table II are collected the results of twelve experiments showing the effect of our high fat diets on the level of blood uric acid. In all cases the amount of uric acid in the blood is increased. The diet previous to the high fat diet was the ordinary hospital diet in all cases but one (R-v-l.) where the fat diet followed immediately on the subject's admittance to the hospital. The height to which the uric acid will rise is sometimes remarkable. J-n-n. at the conclusion of the experiment had 10 mg. of uric acid per 100 cc. of whole blood, though she started at a level of 6.66 mg. Five subjects showed a raised figure of over 6 mg. when the initial level of the uric acid had been 3 or 4 mg. per 100 cc. The effect would appear to be a purely temporary one due to the dietary change, for the uric acid goes back to its original low level, when the fat diet is discontinued, and a carbohydrate or protein diet is substituted. Case S-n-d. is the only exception. Case J-n-n. should not be considered as an exception as labor supervened within 48 hours after the last blood specimen had been taken.

In order to emphasize the effect of this high fat feeding on the level of the blood uric acid we have plotted in Chart 1 the records of two of our subjects, L-n-d. and S-v-g., as these patients were under our observation for a long period of time and we had many opportunities of observing the effect of other kinds of diet. A similar chart over a much longer period of observation may be seen in another publication (9). The charts are almost self-explanatory. There are fluctuations in the uric acid due to diets other than fat such as we have described in a previous paper, but they appear insignificant compared with the changes brought about by

high fat feeding. The charts also illustrate beautifully the rise in non-protein N which very often accompanies the rise in uric acid and which occurs in over 50 per cent of our experiments. The urea N, however, does not show a corresponding increase, the burden of the rise in non-protein N is thus left to the undetermined N fraction.

The increases observed in the uric acid do not occur with equal rapidity in all cases. Thus Le B. (May 26) shows a rapid rise in 3 days, the uric acid being increased nearly 100 per cent in that

TABLE I.

High fat diet No. 1.		High fat diet No. 2.	
	gm.		gm.
Orange.....	60	Grapefruit.....	75
Egg.....	120	Butter.....	40
Butter.....	50	Egg.....	130
Cream.....	175	Cream.....	190
Meat.....	55	Meat.....	55
Potatoes.....	25	10 per cent vegetables....	20
10 per cent vegetables...	20	5 " " ".....	25
Milk.....	105	Gelatin (with saccharine)..	5
Macaroni.....	32	Milk.....	20
Cheese.....	18	Cheese.....	18
Apple.....	100	Apricots or prunes.....	30
Sugar.....	15	Sugar.....	15
Protein.....	45.6	Protein.....	42.1
Fat.....	138.0	Fat.....	104.2
Carbohydrate.....	57.8	Carbohydrate.....	38.1
Calories.....	1,677	Calories.....	1,235

short time, while S-v-g., commencing from almost the same level of uric acid, only increases 50 per cent in 8 days. The increases are undoubtedly due to uric acid and not to some other substance simulating its color reaction with the analytical reagent. The direct method of Benedict for determining uric acid must always be open to a certain amount of suspicion whenever unexpected results are obtained. We have therefore checked our results in the cases of Le B. (May 31) and of L-n-d. (May 31 and June 3) by first carrying out a precipitation by silver lactate, decomposing the precipitated silver salts by NaCl and HCl, and then carrying

TABLE II.
Showing Rise in Blood Uric Acid on High Fat Diets and the Return to a Low Level on a Subsequent Protein or Carbohydrate Diet.

Date.	Subject and calory requirement.	Diet.	Per 100 cc. of blood.				Serum protein.
			Non-protein N.	Uric acid.	Urea N.	NaCl	
			mg.	mg.	mg.	mg.	per cent
1924							
Jan. 3	Ch-t.	Ordinary.	26.0	4.0		495	8.15
" 9		Fat Nos. 1 and 2.	42.5	7.1		485	9.03
" 11	1,715 calories.	" " 1 " 2 + 15 gm. NaCl.*	37.5	7.7		523	7.48
" 16		Carbohydrate.	41.6	4.5		462	7.74
" 7		Admittance.	31.0	2.7		465	6.98
" 11	R-v-l.	Fat Nos. 2 and 1.*	41.0	6.8		472	7.42
" 16	1,633 calories.	Carbohydrate.	39.1	3.1		495	6.45
" 11		Ordinary.	30.0	3.8		516	6.61
" 16	Le B.	Fat No. 1.	33.0	4.4		448	8.19
" 21	1,503 calories.	Carbohydrate.*	21.0	3.1	10.0	519	6.86
" 21		Ordinary.	32.0	3.84		502	7.63
" 24	S-n-d.	Fat No. 1.	43.5	4.69	11.2	492	8.25
" 26	1,598 calories.	" " 1 + 7.5 gm. NaCl.*	38.8	4.76	13.3	508	8.25
Feb. 5		Protein and carbohydrate.	53.0	4.44	14.0	510	7.85
					7.0		
May 26		Ordinary + 3 gm. NaCl.	27.1	3.22	10.9	450	6.41
" 29	Le B.	Fat No. 2 + 3 " "	33.2	6.38	9.3	462	7.37

May 31	1,598 calories.	Fat No. 2	+ 3 gm. NaCl.	35.1	6.27	7.2	6.40
June 3	.	Carbohydrate	+ 3 " "	28.1	3.87	7.4	6.42
May 26	L-n-d.	Ordinary	+ 3 gm. NaCl.	21.3	3.26	7.7	6.34
" 29		Fat No. 2	+ 3 " "	31.2	4.26	9.4	5.68
" 31	1,750 calories.	" 2	+ 3 " "	32.1	5.00	8.2	6.59
June 3		" 2	+ 3 " "	45.0	5.26	8.6	6.51
" 6		Carbohydrate	+ 3 " "†	26.8	4.07	6.8	6.59
" 9		"	+ 3 " "	31.4	3.75	7.4	6.81
May 26	S-v-g.	Ordinary	+ 3 gm. NaCl.	28.3	3.29	10.2	6.49
" 29		Fat No. 2	+ 3 " "	35.7	3.77	12.1	6.94
" 31	1,747 calories.	" 2	+ 3 " "	36.9	4.31	11.6	7.48
June 3		" 2	+ 3 " "	45.8	4.96	12.6	7.85
" 6		Carbohydrate	+ 3 " "†	27.1	3.75	9.8	6.85
" 16	T-l-n.	Fat No. 2	+ 3 gm. NaCl.	44.5	4.25	12.4	9.05
" 19		" 2	+ 3 " "	37.9	4.06	11.6	8.02
" 23	1,705 calories.	Carbohydrate	+ 3 " "	32.4	3.31	7.5	7.31
Apr. 16	Le B.	Ordinary	+ 3 gm. NaCl.	28.1	3.51	14.0	6.03
" 19		Fat No. 2	+ 3 " "	45.0	6.25	8.6	5.90
" 22	1,598 calories.	" 1	+ 3 " "	41.5	6.25	11.0	4.16
" 26		" 1	+ 15 " "	30.5	5.56	7.5	4.16
May 2		Protein	+ 15 " "	28.0	3.03	12.6	5.79

* Moeenthal's nephritic test meal at conclusion of fat diet.

† Water test on first morning of carbohydrate diet.

—Concludes—

Date.	Subject and calory requirement.	Diet.	Per 100 cc. of blood.				Serum protein.
			Non-protein N.	Uric acid.	Urea N.	NaCl	
			mg.	mg.	mg.	mg.	per cent
1924							
Apr. 16	L-n-d. 1,750 calories.	Ordinary + 3 gm. NaCl.	39.1	4.00	9.2	447	6.12
" 19		Fat No. 2 + 3 "	32.2	4.54	9.3	442	5.53
" 22		" " 1 + 3 "	41.5	4.35	8.9	440	5.58
" 26		" " 1 + 15 "	37.5	6.81	8.1	460	5.60
May 2		Protein + 15 "	25.0	2.97	12.1	440	5.79
Apr. 16	J-n-n. 1,684 calories.	Ordinary + 3 gm. NaCl.	45.0	6.66	8.2	445	5.00
" 19		Fat No. 2 + 3 "	42.5	7.69	13.4	448	5.09
" 22		" " 1 + 3 "	36.0	7.69	10.8	445	4.49
" 26		" " 1 + 15 "	38.8	10.00	10.9	460	5.26
Sept. 15	G-h-m. 1,502 calories.	Ordinary + 3 gm. NaCl.	22.0	2.15	13.2	482	6.55
" 19		Milk, cream, and egg white + 3 gm. NaCl.	36.5	3.88	14.2	446	6.94
" 23		" " " " + 15 "	24.7	2.88	10.5	435	7.33
" 27		" " " " + 3 "	25.4	2.85	11.2	446	7.63
Oct. 1		Carbohydrate + 3 gm. NaCl.	18.7	1.70	6.5	462	6.85

out the uric acid determination, using the Benedict reagents. Under these conditions of analysis Le B. (May 31) showed 6.27 mg. of uric acid per 100 cc. of whole blood by the direct colorimetric method, and 5.82 mg. precipitated by silver lactate. L-n-d. gave 5.00 mg. (May 31) and 5.26 mg. (June 3) by the direct method and 4.32 and 5.17 mg. of uric acid precipitated by silver lactate

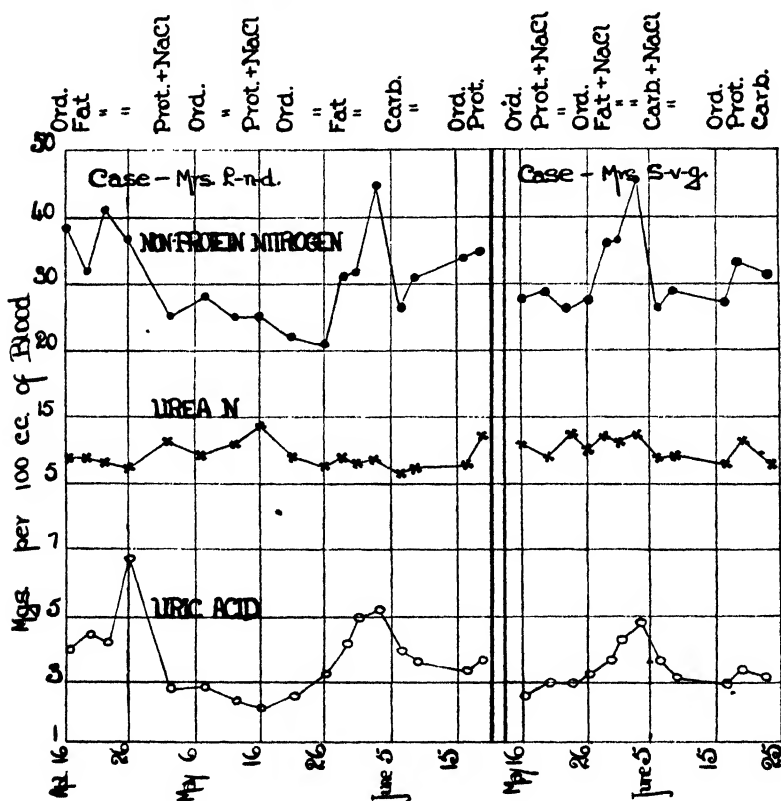


CHART 1.

when on the high fat diet. The direct colorimetric method of Benedict as usual gives a slightly higher result. This increase in the uric acid is circulating in the plasma, for on the same dates, for the same individuals, the plasma showed 7.50, 6.12, and 6.66 mg. of uric acid per 100 cc. respectively.

We have also followed the excretion of uric acid in five cases.

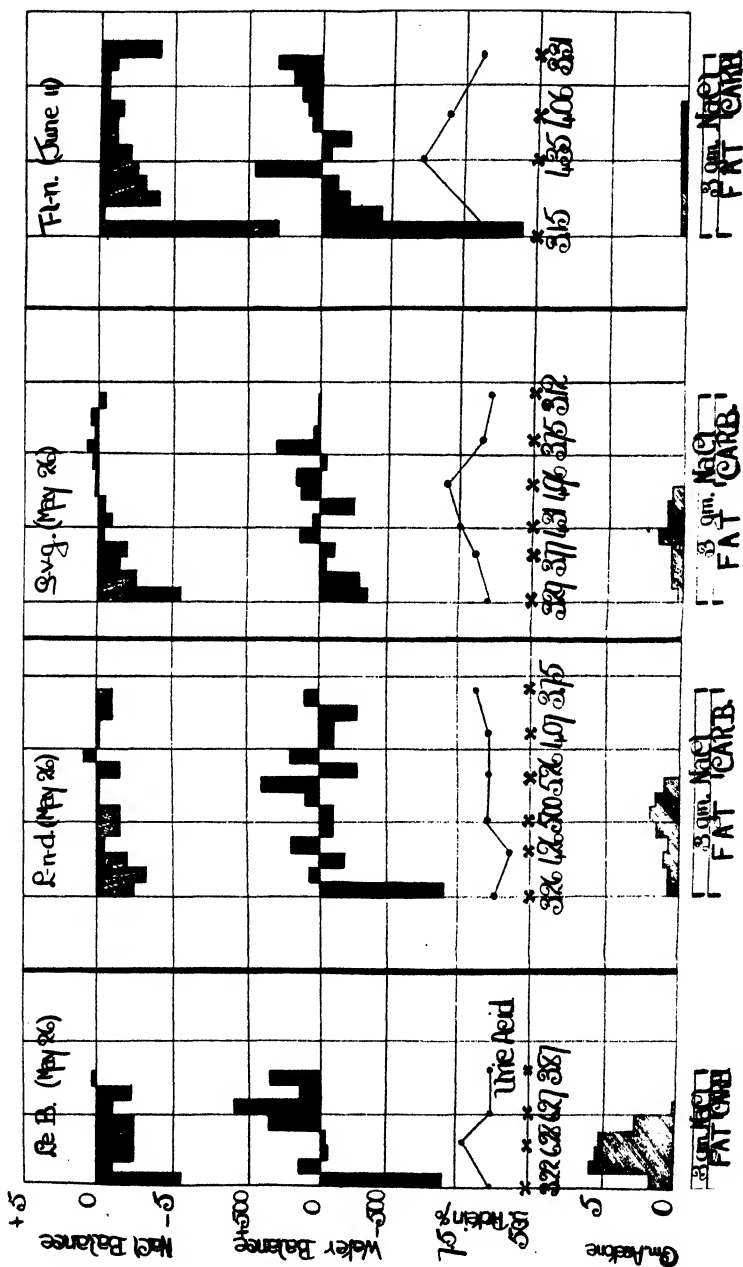
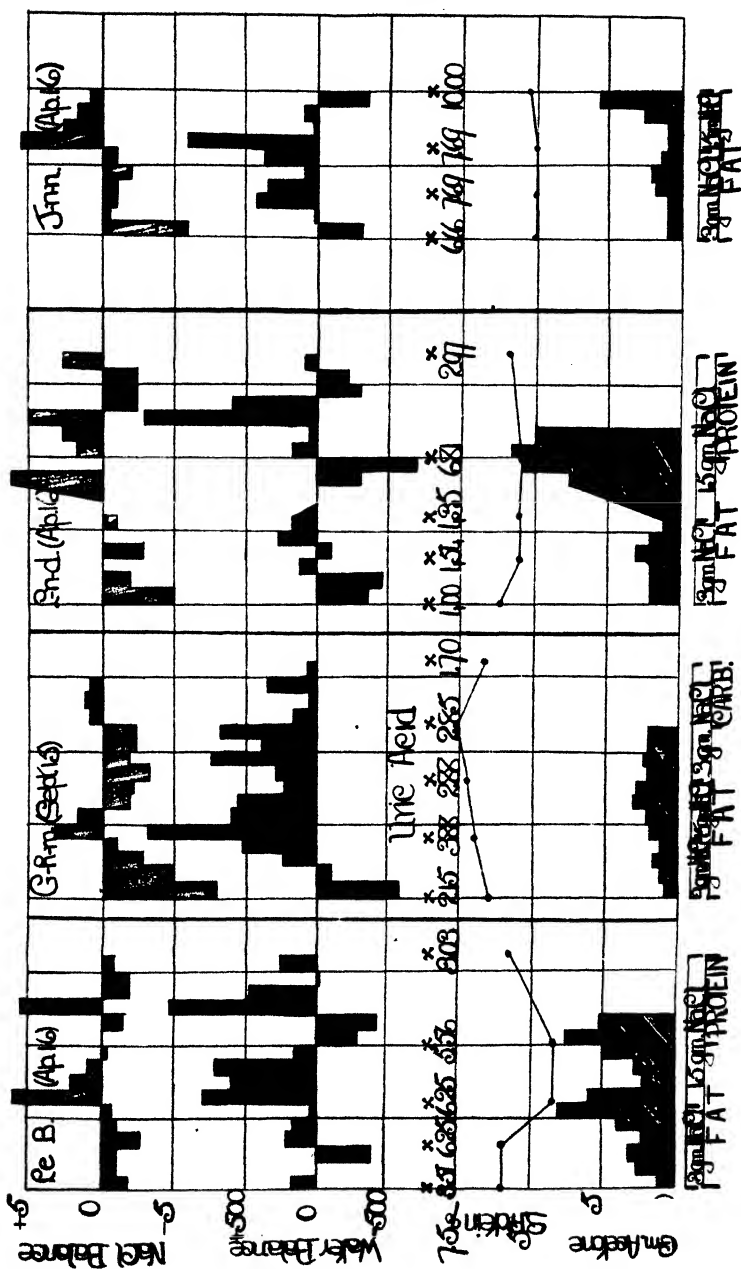


CHART 2.



CHART

Three of these cases are those whose blood uric acids we have just discussed in detail when the high fat diet is followed by a carbohydrate diet. Both diets contained small amounts of purines from meat. In G-h-m., however, all diets were purine-free. The noticeable feature is that at the beginning of the carbohydrate diet there is a marked increase in the excretion of uric acid. This amount is greater than on the high fat diet for some days previous, or on the carbohydrate diet itself, and we should infer that it represents part at least of the accumulated uric acid in the blood. These results are shown in Table III.

TABLE III.

Showing the Daily Excretion of Uric Acid on Fat Diet Followed by Carbohydrate Diet.

Uric acid in 24 hrs.					Diet.
Le B.	L-n-d.	S-v-g.	T-l-n.	G-h-m.	
	478	542	477		Fat.
	382	509	460		
	410	534	629		
410	290	489	374		
240	332	307	245	239	
350	400	398	245	268	
354	304	376	394	260	
435	378	472	372	308	
					Carbohydrate.
484	511	660	564	315	
550	449	554	420	382	
426	352	304	514	300	
	392	470	320	350	
	450	441			
	440	423			

DISCUSSION.

The results we have just given make it very plain that on a high fat diet such as we have used the blood uric acid will be increased. The increase may occur slowly or rapidly, and the height to which the uric acid may rise may vary in individual cases, but if the experiment be continued long enough it will become evident. A carbohydrate diet will rapidly reduce the high level of the blood uric acid to a normal range of figures, which

reduction is accompanied by an increased elimination into the urine for the first 24 or 48 hours. A protein diet will also bring the raised blood uric acid down to a normal level. Decreased elimination might thus appear to be the explanation of the high values in the blood.

The literature has many experiments from which it would appear clear that high fat diets, and particularly those sufficiently high in fat to produce ketosis, lead to a decreased elimination of uric acid into the urine. Thus Cathcart (10) in 1909 observed that changing from a carbohydrate-rich fat-poor diet to a carbohydrate-poor fat-rich diet led to a decreased elimination of uric acid, and Graham and Poulton (11) noted a marked diminution in the uric acid excretion in all conditions associated with a high fat diet, though they discard this fact as the actual reason. Umeda's experiments (12) too are convincing, for on a diet of adequate and constant calories, and constant protein and salt intake, the uric acid excretion varies inversely with the percentage of calories arising from fat. Starvation, too, where the body draws the greater proportion of its calories from fat, is marked by a diminution in uric acid elimination, especially in the first few days, although Cathcart (13) showed that later in the starvation the uric acid rises to its former level. The observations of Lennox (1) show that blood uric acid values are increased in starvation.

It is common to ascribe high blood uric acid values to some failure of kidney function, but nephritic test meals, or water tests, carried out on some of our subjects when showing high blood uric acid values, gave normal results. Moreover, the obviously transient nature of the phenomenon and its dependence on dietary changes preclude its origin in a damaged kidney and turn our attention in search for the reason to extrarenal causes.

The simplest of such explanations would be decrease in blood volume, leading to decreased capillary flow and hence decreased elimination through the kidneys. Such a point of view would harmonise with that expressed in a previous paper on the influence of sodium chloride on blood uric acid. There, it was pointed out that sodium chloride decreased the level of blood uric acid most probably by increasing blood volume and thus increasing elimination. This view would make the action of a high fat diet the opposite in character to a high salt diet. There is much to be said in

favor of such a view. Thus Benedict and Milner (14) showed that, on isodynamic diets, gains in water occurred when 66 per cent of the energy arose from carbohydrate, but that marked losses in water were noticed when 67 per cent of the energy arose from fat. Gamble, Ross, and Tisdall (15) in a study of starvation have confirmed the earlier findings that there is a depletion of the body cells of water in the first 24 or 48 hours. Some other experiments of our own have shown that the concentration of serum proteins in dogs is markedly increased on a high fat diet. By thus endeavouring to link up decreased elimination and increased blood values of uric acid on high fat diets by means of changes in blood volume, it is evident that we have adopted, in part, the view expressed by Folin, Berglund, and Derick (16) that many of the inconsistencies of endogenous uric acid metabolism might be explained by variations in the concentration in the blood.

In searching for evidence in support of such an expectation that changing blood volumes would prove to be the explanation of our results, we have examined our records for fluid intake and urine output, for sodium chloride balances, and for the concentrations of serum proteins. We have presented in Charts 2 and 3 the findings in our later experiments. The earlier ones are marred from this point of view by the introduction of a nephritic test meal after the high fat diet. In general, however, they show the same characteristics. The charts show also the corresponding levels of blood uric acid, and the excretion of total acetone bodies in the urine. The determination of the water and NaCl balances in this way we know to be only approximate. Nevertheless, we take it that if we observe at the beginning of the high fat feeding, when we are changing from an ordinary mixed diet, a rush of water and NaCl into the urine in contrast with later periods, or on the ensuing carbohydrate or protein diet, it is evidence of a change of concentration of body water. If this, in addition, is coupled with the observation of increase of serum protein concentration, we would infer that the blood volume has undergone a diminution.

All the experiments show a loss of NaCl and water into the urine at the beginning of the high fat diet. This is very marked in T-1-n. (non-pregnant), probably because she came to us from another ward where she had been on an unrestricted salt régime. In all the other cases the previous diet had contained 3 gm. of

NaCl so that the effect observed is due to the change to a fat diet and not to any alteration in the intake of NaCl. Of the eight experiments represented in these charts, however, a rise in the concentration of serum proteins is observed only in four cases coincident with the reduction of water volume (Le B., May 26, S-v-g., T-l-n., G-h-m.). There may possibly be a slight rise in the case of L-n-d. (May 26). The change from the fat diet to either a carbohydrate or protein diet, which is accompanied by a reduction of the blood uric acid, is marked by a decrease in the concentrations of serum proteins in the same four cases. There is too a reversal of the general trend of the water balances on the change of diet, though losses of NaCl are still to be noted when the diet only contained 3 gm. of salt. The introduction of 15 gm. of NaCl into the high fat diet is shown in Chart 3. Its immediate effect is to cause a retention of sodium chloride and water. The serum proteins remain at about the same level and the blood uric acid shows a very marked decrease in Le B. (April 16) and G-h-m., as might be predicted from our previous paper, but J-n-n. and L-n-d. (April 16) show an increased amount of blood uric acid despite the retention of water and salt. The change to a protein diet in the cases of Le B. and L-n-d. with the same intake of 15 gm. of NaCl is, however, promptly followed by a large retention of water and salt and a rapid drop of the uric acid to normal figures.

The evidence then is not as clear as we should wish. There is an undoubted loss of water and NaCl at the beginning of the diets, and in all cases an increase in the level of blood uric acid is observed, even in the short space of 3 days. The only doubt which may be raised is due to the behaviour of the serum proteins. These do not invariably increase in concentration. In spite of this, however, we believe that at least part of the increase in the blood uric acid observed in our high fat diets is brought about by a decrease in blood volume, and hence is due partly to decreased elimination. It is the increased elimination of uric acid in the urine on the 1st day of a carbohydrate diet following the fat diet which makes us adopt this attitude. Combined with it is also the fact that the determination of the total serum proteins by a single refractometer reading is always open to error in the presence of any marked change in the albumin-globulin ratio.

Although we have thus indicated our belief that a reduction in

blood volume is responsible for the rise in blood uric acid, thus making the effect of a high fat diet opposite in character to that of NaCl, yet an inspection of our figures reveals that other factors must come into play. It is in the magnitude of the changes that this becomes apparent. In all, we have a record of twelve experiments, and of these, six show increases in the level of blood uric acid far greater than we should have expected, were the changes simply brought about by an altered level of excretion. The cases referred to are those of Ch-t., R-v-l., Le B. (April 16, May 26), L-n-d. (April 16), and J-n-n. (the two last cases after the addition of NaCl to the diet). The blood uric acid is characterised by an abrupt rise of considerable magnitude. The maximum values are rapidly attained and reach distinctly pathological figures; *viz.*, 7.08, 6.78, 6.25, 6.28, 6.81, and 10.0 mg. per 100 cc. of blood. 3 or 4 days of a carbohydrate or protein diet reduces them to normal figures; *viz.*, 4.47, 3.12, 3.03, 3.87, and 2.97. (In the sixth case it was not possible to examine the effect of a carbohydrate or protein diet). The maximum values in the other cases are 4.45, 4.69, 5.26, 4.96, 4.06, and 3.88 mg. per 100 cc. of blood, a series of figures markedly less than those of the previously mentioned group, and in two of these cases (L-n-d., May 26 and S-v-g.) the fat diet was continued for 8 days. The effect of the ensuing carbohydrate or protein diet is also less abrupt, giving figures of 3.11, 4.06, 3.75, 3.12, 3.31, and 1.70 mg. per 100 cc. of blood. With the exception of the last figure the range of values on the carbohydrate or protein diet is the same in both series. Moreover, in the last case just mentioned the figure 1.70 mg. of uric acid per 100 cc. of blood (G-h-m.) had been attained from a previous figure of 2.88 mg. on the fat diet, and not from the maximum of 4.88 mg., the value on the fat diet having been lowered by the use of NaCl. In addition it may be mentioned that in cases L-n-d. (May 26) and S-v-g., where 8 days had been spent in raising the blood uric acids from 3.26 and 3.29 mg. to 5.26 and 4.96 mg., respectively, that 6 days were required to lower them to the levels 3.75 and 3.12 mg. by means of carbohydrate.

These changes appear to us as so distinct in the two sets of cases just mentioned that we cannot help but examine them for the presence of some other factor or factors which might augment that of decreased blood volume and thus raise the value of the uric

acid. The diets we used were calculated to contain such proportions of carbohydrate, protein, and fat, as to be on the border line of acetone production, on the assumption that 1 molecule of antiketogenic material is requisite for the complete combustion of 1 molecule of ketogenic substance. Acetone was produced in all cases, sometimes in large amounts, and it is in this latter fact that we believe we see an augmenting factor in the production of the raised blood uric acid values. The amounts of "total acetone" are shown on Charts 2 and 3 and what we have noted is that the abrupt rises in blood uric acid values have always occurred in the presence of a high ketonuria, usually above 2 or 2.5 gm. per day. This is particularly noticeable in the cases of L-n-d. (April 16) and J-n-n. The increases in blood uric acid were marked, but not remarkably large on the high fat diet, until the introduction of 15 gm. of NaCl when they rose from 4.35 to 6.91 mg. in the case of L-n-d. and from 7.69 to 10.00 in the case of J-n-n. At the same time, the acetone excretion increased from an average of slightly over 1 gm. to 10 gm. in the first case and 5 gm. in the second. None of the cases who showed a slow rise in blood uric acid showed an acetone excretion of over 2 gm., while those cases in whom the rise of blood uric acid was abrupt showed excretions of acetone varying from 3 to 8 gm.

We have thus been led to connect the height of the acetone excretion and the rise in the blood uric acid. Why there should be this connection is not at all clear. We hardly think that the connection is a direct one, and that the actual presence of the acetone bodies in the blood has an immediate influence on the level of the blood uric acid, although the experiments of Gibson and Doisy (17) are suggestive on this point. Nor would it appear from the experiments of Folin, Berglund, and Derick (16), on the behaviour of injected uric acid in the depancreatized dog, that disturbances in carbohydrate metabolism, such as might account for acetone production, are responsible for the abrupt rises in blood uric acid. Disturbances in ionic balance might be considered, but we do not wish to hazard any guess as to its nature at the present juncture. Only when we have fuller experiments on the conditions of production of both acetone bodies and uric acid will it be worth while to elaborate any theory.

We have also noted that sometimes there occurs an increase in

the non-protein N concomitant with the high fat diet, without any rise in the urea content of the blood. This is to us a matter of great interest, as increases of such a nature have only been observed hitherto in pathological conditions. Thus Killian and Sherwin (18) have noted increase in the undetermined blood N in some of the "toxemias" of later pregnancy, a fact confirmed by this laboratory. We have also noted, however, that such increases may be met with in a normal pregnancy. Killian (19) has also noted rises in the non-protein N, not attributable to urea, in pneumonia before the crisis. Here we have a similar change brought about by a dietary influence in a comparatively easy manner, and thus susceptible to experimental control and investigation.

SUMMARY.

1. Diets sufficiently high in fat to produce ketosis result in an increase in the uric acid content of blood.
2. A carbohydrate or protein diet lowers blood uric acid, raised by a high fat diet, to its normal level.
3. The increases in blood uric acid produced by a high fat diet can partly be accounted for by a decreased blood volume with a consequent decreased elimination.
4. In 50 per cent of the cases, however, such an explanation would appear insufficient to account for the magnitude of the increase in the blood uric acid.

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THE ADSORPTION OF INDICATOR (CRESOL RED) BY SERUM IN THE SPECTROPHOTOMETRIC DETER- MINATION OF THE pH.*

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The spectrophotometric determination of the H ion concentration of liquids as described by Brode (1) and by Holmes (2), if applicable, would be a convenient method for making such measurements of blood plasma. According to their work, a change in the reaction of a liquid containing an indicator is accompanied by a change in the height and not by a shifting of an absorption band when the solution is examined with a spectrophotometer at a characteristic wave-length. With a two-color indicator the height of the absorption bands can be measured at one or both of the characteristic wave-lengths. The readings obtained with buffer mixtures of known pH value, and containing a definite concentration of indicator, may be used as a scale for converting into equivalent pH values other readings made in the same way of similar unknown solutions.

In order to determine whether the spectrophotometric method could be used in measuring the pH of serums or plasmas, cresol red as indicator was chosen because the usual variations in the reaction of the blood are within the middle and lower portions of the pH range (6.4 to 9.4) of the indicator. 0.1 gm. of cresol red was dissolved in 5.3 cc. of 0.05 N NaOH and distilled water enough to make a total volume of 500 cc. (3). Buffer solutions for the pH range 6.8 to 7.8 were prepared (3), and their hydrogen ion concentrations were determined electrometrically. By trial experiments, using a Keuffel and Esser color analyzer, and having the solutions contained in an analyzer tube 1 cm. long, the proper

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amount of indicator solution to be added to 3 cc. of the buffer solution was determined. This was found to be 0.26 cc., an amount which with the color analyzer mentioned gave readings of 0 to 100 per cent over the pH scale of the indicator, with the measurements made at wave-length $572 m\mu$. For the range pH 7.0 to 7.7 the results obtained are given in Chart 1. They agree with the percentage transmission values given for cresol red by Brode (1).

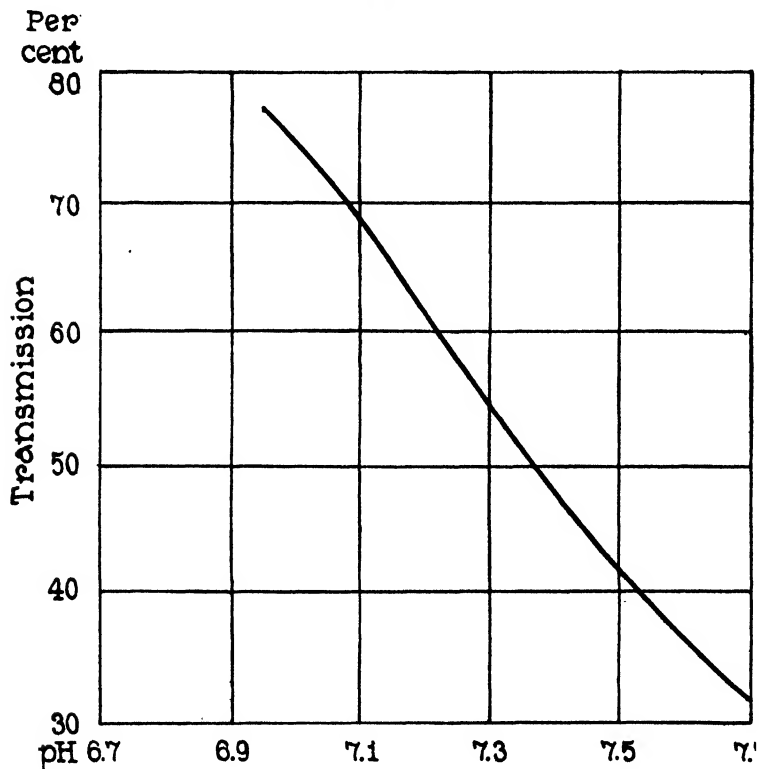


CHART 1.

Having established the amount of indicator solution necessary for the buffer mixtures mentioned, experiments were made with serum. Human, rabbit, and sheep serums obtained from clotted blood were acidified slightly with 0.1 N HCl in order to bring the reaction within the pH range 6.8 to 7.8. The H ion concentration was measured electrometrically with one portion. Quanti-

TABLE I.

Serum.	Electrometric pH.	Quantity of indicator solution.	Transmission.	Equivalent pH.
		<i>cc.</i>	<i>per cent</i>	
Rabbit (pooled).	7.38	0.20	56	
		0.24	51	
		0.26	49	7.38
" (")	7.29	0.26	56	7.28
	7.30	0.26	55	7.29
	7.10	0.26	66	7.13
Human (")	7.52	0.38	47	
		0.40	42	7.49
" (")	7.42	0.40	47	7.40
	7.14	0.40	64	7.16
"	7.43	0.40	53	
		0.50	46	7.42
"	7.35	0.40	50	7.35
		0.50	44	
"	7.35	0.68	49	7.37
"	7.33	0.68	50	7.35
" (")	7.62	0.68	35	7.62
	7.35	0.68	53	7.34
	7.22	0.68	60	7.22
	7.01	0.68	76	6.98
Sheep.	7.28	0.4	83	
		0.9	71	
		1.2	58	7.24
"	7.55	1.6	25	
		1.3	28	
		0.9	39	7.54
"	7.63	1.2	47	
		1.6	35	7.62

ties of the indicator solution were added to other 3 cc. portions of serum until the percentage transmission measured at wave-length $572m\mu$ agreed with the percentage values obtained with the standardized buffer mixtures. The results obtained are collected in Table I. The control tube in the spectrophotometer contained samples of serum at the same pH as that with the indicator, and diluted with a volume of distilled water equal to the indicator solution added.

According to these results the amount of indicator in serum necessary to make the percentage readings of the spectrophotometer fall on a curve representing pH values is not the same regularly, as that in the ordinary buffer solutions. With rabbit serum the amount is about the same, but with human serum it is more, while with sheep serum the quantity is considerably greater. The results also show that in different serums of the same species, the amount of indicator necessary is not the same, but if the proper amount of indicator is determined with a given sample of serum for any part of the pH scale studied, the values in other parts can be determined with accuracy. When the H ion concentration of fresh serum or plasma is being determined under conditions where CO_2 loss must be avoided and preliminary tests for determining the proper amount of indicator are impracticable, this variation in the amount of indicator necessary for proper percentage readings makes doubtful the accuracy of a direct transposition into equivalent pH values.

The variation in the amount of indicator in serum necessary to make the proper transmission value for a given reaction suggests that a certain portion of the indicator is used up, or bound in some way by substances contained, and that after this affinity has been satisfied there must be the proper amount of indicator unbound in the solution to undergo the tautomeric transformations incident to the changes in reaction of the liquid. It may be that the difference in the amount of indicator necessary is due to the protein content of the serum. This statement alone is not satisfactory, for rabbit serum which requires about the same amount of indicator as the ordinary buffer solutions, contains protein substances in high concentration. Inorganic substrates also can bind dyes (4), but the variations in the inorganic content of rabbit, human, and sheep serums are not sufficient to explain the differ-

ences observed. The quantity of indicator bound by the serum of a certain species may be related with a specific protein content.

CONCLUSION.

The amount of indicator (cresol red) necessary for correct transmission values with the spectrophotometric determination of the H ion concentration of serums varies for different species and even with serums of individuals from the same species.

The amount in rabbit serum is approximately the same as that necessary in the ordinary buffer solutions.

A certain amount of the indicator seems to be bound by some of the serum constituents. When this affinity has been satisfied, or an equilibrium established, there is needed a definite amount unbound which undergoes the tautomeric changes incident to pH variations.

These differences in the quantity of indicator bound by serums may be associated with species specificity.

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ON THE MECHANISM OF WATER INTOXICATION.

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(Received for publication, December 6, 1924.)

It has been demonstrated by Rowntree¹ that the administration of large volumes of water by mouth to dogs and other laboratory animals induces an intoxication that may lead to convulsions and death. On the other hand, similar volumes of isotonic salt solution fail to provoke any of the significant manifestations of water intoxication. Hypertonic salt solution given intravenously prevents the onset of convulsions and coma, and causes rapid clinical improvement after toxic symptoms have been induced. Necropsy findings are essentially negative. Study of the mechanism responsible for the abnormal symptoms evoked leads to the conclusion that definite changes in blood composition occur. Thus, the ingestion of the large volumes of water causes significant dilution of the blood and of the serum proteins. The salt content is also reduced, the chlorides and sodium being decreased to an extent much greater than might be expected from the degree of dilution. The urinary chlorides are only slightly increased.²

The character of the symptoms manifested, together with the fact reported by Rowntree that physiological salt solutions fail to elicit the typical syndrome, naturally leads to the query whether the passage through the body of such large volumes of water may rob the organism of its salt content to such an extent that a resultant upset of the water-salt equilibrium occurs.³

To determine the extent to which substances may be washed out of the body, water has been administered to dogs to the point of intoxication, and appropriate analyses of the urine and blood have been made.

¹ Rowntree, L. G., *Arch. Int. Med.*, 1923, xxxii, 157.

² Greene, O. H., and Rowntree, L. G., *Am. J. Physiol.*, 1924, lxviii, p. iii.

³ This investigation was carried through in the early part of 1923, nearly a year before the experiments of Greene and Rowntree² were reported.

Methods.

Female dogs, in a fasting condition, were given by means of a stomach tube 50 cc. of water per kilo of body weight at intervals of $\frac{1}{2}$ hour until definite evidences of toxicity had been induced. No attempt was made to bring about death. The animals were maintained in metabolism cages, the urine being separated into 24 hour specimens by catheterization. Urinary constituents were determined as follows: chlorides (Volhard-Harvey), phosphates (uranium titration), total acidity (Folin), ammonia (Folin), creatinine (Folin), and creatine (Folin-Benedict). Changes of hemoglobin content (Cohen and Smith), and, in some instances, alterations of chloride content (Whitehorn) were followed in blood, drawn from the external jugular vein.

Alterations in Blood and Urine Composition during Water Intoxication.

The experimental results are shown in the accompanying protocols and tables. Table I gives the analysis carried through in the experiment of which Protocol I is descriptive. A similar numerical relationship holds for the other tables and protocols.

In all cases the earliest evidences of toxicity—restlessness, salivation, and vomiting—appeared when water had been administered to about one-third the initial body weight. For the more pronounced toxic manifestations it was found necessary to give almost two-thirds the body weight of water. The forcing of water was discontinued when definite symptoms appeared; and all the dogs recovered.

From the tables it may be seen that the urine volume paralleled the water intake and that in all instances there were found marked and consistent increases in the excretion of chlorides, phosphates, ammonia, and total acid. In those cases where creatinine was determined a similar statement may be made. Creatine was increased also, but to a less marked extent. The urinary excretion returned to the normal level soon after cessation of water-forcing, indicating the absence of outspoken renal damage.

Accompanying the course of water introduction there was progressive decrease in the hemoglobin concentration, beginning in most instances at about the time when first evidences of toxicity

were noted. At the height of the intoxication the hemoglobin content reached its lowest level, usually showing a decrease of 10 to 15 per cent, the initial value being taken as 100 per cent. In those instances where determined the chlorides of the total blood showed a similar change which, however, was too great to be accounted for by mere dilution of the blood.

In no experiment was subcutaneous edema observed. The elimination of fluid was rapid and although increases in body weight were significant the change was merely transitory. The significance of the gain in body weight is difficult of interpretation since it was impossible to determine how much of the gain represented water in the gastrointestinal tract not actually absorbed at the time of weighing.

The results of the present investigation lend support to the hypothesis that water intoxication may be explained by disturbance of osmotic relationships in the tissues. The pronounced increase in the excretion of the urinary constituents determined indicates that there must be a very considerable loss through the urine of inorganic ions. Inasmuch as increased elimination occurred whenever tested, it may be assumed that augmented output of other undetermined urinary constituents also took place. Further evidence of loss of salt to the body is the marked diminution in the blood chlorides.

CONCLUSIONS.

In the condition of water intoxication there is a marked increase in the elimination of urinary chlorides, phosphates, total acid, ammonia, and creatinine.

The blood shows definite and consistent dilution, most marked at the height of the intoxication.

Blood chlorides are diminished to a degree greater than can be accounted for by mere dilution of the blood.

The fluid is rapidly excreted since the urine volume almost parallels the water intake.

The animals quickly recover, and the urine and blood compositions rapidly return to normal with cessation of water administration.

The loss of tissue salts with consequent disturbance of the water-salt equilibrium of the body constitutes an important factor in the mechanism of water intoxication.

PROTOCOL I.
Induction of Water Intoxication in Dog D.

Date.	Time.	Weight.	Water intake.	Hemo- globin in percent- age of normal.	Remarks.
1923	a.m.	kg.	cc.		
Feb. 9	9.15	13.2		100	Catheterized.
	9.30		600		
	10.00		600		
	10.30		600		
	11.00		600	96.4	
	11.30		600		
	m				
	12.00		600		
	p.m.				
	1.00	14.25	600	93.8	Restlessness, nausea, vomiting.
	1.30		600		
	2.00		600		Twitching. Ataxia.
	2.30	14.6	600	88.6	Unable to stand. Water dis- continued. Total intake 6,000 cc.
	2.37				Choreiform movements of head. Muscle twitchings increasing in severity.
	2.40				Involuntary urination followed by violent clonic convulsions of entire body. Convulsions became tonic. Opisthotonos. Vomiting.
	3.15				Lies motionless on side. Pant- ing. Typical Cheyne-Stokes breathing.
	3.30				Many feeble attempts to rise. Falls heavily against side of cage. Lower jaw drops. Sal- ivation and tremor marked.
	4.15				Excitement. Threshes about. Whining. Manages to get up by clinging to cage walls.
	4.25				Lies on side quietly. Flaccid. Stuporous.
	4.35	14.2		93.0	Unconscious.
	6.00	13.8			Can stand. Weak. Restless.
	a.m.				Feeble barking.
Feb. 10	9.15	13.4		99.5	Toxic. Dull. Weak. Catheter- ized.
" 11	9.15	13.0		103.2	Normal. Hungry. Catheterized.

TABLE I.
Urinary Analysis during Water Intoxication in Dog D.

Date.	Water intake.	Urine volume.	Specific gravity.	Chlorides as NaCl.	Phosphates as P_2O_5 .	Ammonia nitrogen.	Total acidity as NaOH.	Creatinine.	Creatinine.
1923	cc.	cc.		gm.	gm.	mg.	gm.	mg.	mg.
Feb. 9	230	140	1.040	0.15	1.124	180	0.84	92.5	13.1
" 10	6,000	5,400	1.005	4.23	3.060	547	2.82	204.2	37.0
" 11	75	190	1.035	0.16	1.480	620	1.04	116.6	21.3
" 12	175	155	1.043	0.09	1.745	360	1.22	115.2	21.5
" 13	200	160	1.042	0.07	1.310	730	0.74	108.4	

PROTOCOL II.
Induction of Water Intoxication in Dog Alpha.

Date.	Time.	Weight.	Water intake.	Blood.		Remarks.
				Hemoglobin in percentage of normal.	Chlorides as NaCl per 100 cc. blood.	
1923	a.m.	kg.	cc.		mg.	
Jan. 21	9.00	9.7		100	310	Catheterized.
	9.15		500			
	9.45		500			
	10.15		500			
	10.45		500			
	11.15	10.52	500	95.8	280	Vomiting.
	11.45		500	95.3		Salivation, restlessness.
	p.m.					
	12.15		500	95.5		Muscle twitchings, vomiting.
	12.45		500	92.1		Tremor. Salivation greatly increased, apathy, head droops.
	1.30		500			Ataxia, weakness, cannot stand.
	2.00		500	87.4	230	Clonic followed by tonic convulsions, stuporous, breathing irregular. Water discontinued.
	5.30		10.3	94.2	280	Weak. Lies quietly. Conscious, toxic.
	a.m.					
Jan. 22	9.00	9.65		103.6	325	Catheterized. Normal.
" 23	9.00	9.60		102.8	320	" Condition good.

TABLE II.
Urinary Analysis during Water Intoxication in Dog Alpha.

Date.	Water intake.	Urine volume.	Specific gravity.	Chlorides as NaCl.	Phosphates as P_2O_5 .	Ammonia nitrogen.	Total acidity as NaOH.	Creatinine.	Creatinine.
1923	cc.	cc.		gm.	mg.	mg.	gm.	gm.	mg.
Jan. 20	270	250	1.043	0.170	0.683	287	0.360	147.5	18.30
" 21	5,000	4,300	1.004	2.830	2.650	497	1.376	219.0	62.75
" 22	80	235	1.027	0.050	0.758	345	0.688	122.5	34.30
" 23	150	110	1.035	0.125	0.916	211	0.728	128.8	28.10

PROTOCOL III.
Induction of Water Intoxication in Dog B.

Date.	Time.	Weight.	Water intake.	Blood		Remarks.
				Hemoglobin in percentage of normal.	Chlorides as NaCl per 100 cc. blood	
1923	a.m.	kg.	cc.		mg.	
Jan. 30	10.00	10.35		100	352	Catheterized.
	10.10		500			
	10.40		500			
	11.20		500			
	11.50		500			
	p.m.					
	12.20		500			
	12.50		500	96.2	315	Vomited.
	1.20		500			"
	1.50	10.81	500			Salivation, appears to be in pain, trembling.
	2.15		500	93.0	281	Salivation increased. Vomiting.
	2.45		500			Stuporous, ataxia, frothing at mouth.
	3.10		500			Increased activity, ataxia more marked.
	3.45	11.5	500	92.6	234	Water discontinued. Generalized convulsions.
	5.00					Quiet. Stuporous.
	a.m.					
Jan. 31	10.00	10.2		101.8	348	Appears to be normal. Catheterized.
Feb. 1	10.00			102.2	340	Hungry. Catheterized.

TABLE III.

Urinary Analysis during Water Intoxication in Dog B.

Date.	Water intake.	Urine volume.	Specific gravity.	Chlorides as NaCl.	Phosphates as P_2O_5 .	Ammonia nitrogen.	Total acidity as NaOH.	Creatinine.
1923	cc.	cc.		gm.	gm.	mg.	gm.	mg.
Jan. 30	190	175	1.026	0.165	0.760	63	0.550	120
" 31	6,000	4,850	1.003	1.890	2.360	219	1.870	232
Feb. 1	60	130	1.022	0.120	0.605	72	0.472	64
" 2	140	125	1.025	0.450	0.633	77	0.520	87

PROTOCOL IV

Induction of Water Intoxication in Dog C.

Date.	Time.	Weight.	Water intake.	Hemoglobin in percentage of normal.	Remarks.
1923	a.m.	kg.	cc.		
Jan. 31	9.45	8.56		100	Catheterized.
	10.00		450		
	10.30		450		
	11.00		450		
	11.30		450		
	m.				
	12.00		450		
	p.m.				
	1.00		450		
	1.30		450		
	2.00		450		Vomiting.
	2.30		450	93.4	" Trembling.
	3.00		450		Salivation, muscle twitching.
	3.30		450		Vomiting.
	4.00		450		" choreiform movements, ataxia.
	4.30		400	90.9	Generalized convulsions, stupor, water discontinued.
	a.m.				
Feb. 1	9.45			98.2	Catheterized. Normal. Weak. Walks about cage. Will not eat.

TABLE IV.
Urinary Analysis during Water Intoxication in Dog C.

Date.	Water intake.	Urine volume.	Specific gravity.	Chlorides as NaCl.	Phosphates as P_2O_5 .	Ammonia nitrogen.	Total acidity as NaOH.
1923	cc.	cc.		gm.	gm.	mg.	gm.
Jan. 31	200	175	1.026	0.085	0.40	70.5	0.24
Feb. 1	5,800	4,500	1.002	2.320	1.67	192.7	1.88
" 2	50	250	1.015	0.530	0.51	39.2	0.32

PROTOCOL V.
Induction of Water Intoxication in Dog E.

Date.	Time.	Weight.	Water intake.	Blood.		Remarks.
				Hemoglobin in percentage of normal.	Chlorides as NaCl per 100 cc. blood.	
1923	a.m.	kg.	cc		mg.	
Feb. 16	8.45	11.87		100	262	Catheterized.
	9.00		600			
	9.30		600			
	10.00		600			
	10.30		600			
	11.00		600			
	11.30		600	95.5		Vomiting, diarrhea.
	m					
	12.00		600			Salivation, twitching, listless.
	p.m.					
	12.30		600	97.4		Weakness.
	1.00		600			Restlessness.
	2.00		600	94.2		Ataxia. Salivation marked, cannot stand.
	2.30		600			Marked tremor, choreiform movements.
Feb. 17	3.00		600			Convulsive movements, stuporous.
	3.30	13.1	600	91.9	210	Generalized convulsions. Water discontinued.
	5.00					Unconscious.
	a.m.					
	8.40	12.3		97.2	246	Catheterized. Weak. Conscious. Dull.
" 18	8.45	11.95			271	Catheterized. Normal. Appetite poor.

TABLE V.
Urinary Analysis during Water Intoxication in Dog E.

Date.	Water intake.	Urine volume.	Specific gravity.	Chlorides as NaCl.	Phosphates as P_2O_5 .	Ammonia nitrogen.	Total acidity as NaOH.	Creatinine.
1923	cc.	cc.		gm.	gm.	mg.	gm.	mg.
Feb. 16	150	125	1.041	0.08	0.86	54.0	0.824	120.0
" 17	7,800	6,200	1.005	3.23	2.30	222.0	3.440	218.0
" 18	50	250	1.020	0.04	0.71	43.2	0.740	124.8
" 19	100	200	1.018	0.72	0.92	72.4	0.790	66.7

PROTOCOL VI.
Induction of Water Intoxication in Dog F.

Date.	Time.	Weight.	Water intake.	Hemoglobin in percentage of normal.	Remarks.
1923	a.m.	kg.	cc.		
Feb. 27	8.30	8.7	450	100	
	9.00		450		
	9.30		450		
	10.00		450		
	10.30		450		
	11.00		450		
	11.30		450		
	m.				
	12.00	8.7	450	95.6	Vomiting.
	p.m.				
	12.30		450		" Marked salivation.
	1.00		450		Ataxia, restlessness, tremor.
	1.30		450	94.2	Respiration shallow and irregular.
	2.00		450	94.7	Toxic symptoms, but no convulsions. Water discontinued.
	a.m.				
Feb. 28	8.30				Catheterized. Normal.

TABLE VI.
Urinary Analysis during Water Intoxication in Dog F.

Date.	Water intake.	Urine volume.	Specific gravity.	Chlorides as NaCl.	Phosphates as P_2O_5 .	Creatinine.
1923	cc.	cc.		gm.	gm.	mg.
Feb. 27	100	80	1.038	0.11	0.56	76.8
" 28	5,400	4,750	1.003	3.42	1.08	108.0
Mar. 1	50	250	1.026	0.08	0.67	84.3

A PROTEIN IN THE EDIBLE PORTION OF ORANGE.

PRELIMINARY PAPER.

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The growing importance of the citrus fruits, especially oranges, in the dietary and in therapeutics naturally invites closer scrutiny into their chemical make-up. Previous investigations have dealt with the kind and amount of acids, the nature of the sugars, the extraction and behavior of the pectin, and with the value of orange juice as a source of vitamins; but data concerning the nitrogenous constituents, especially the presence and character of protein in orange juice, are not available. That the investigation of this group of substances in the juice of plant leaves and fruits is practically a virgin field for research is apparent from the published results of the pioneer work begun by Osborne and his associates. The unusual and varied character of the compounds already isolated in these experiments indicates that through them we are probably approaching a new understanding of the nature of the intermediate compounds produced and, therefore, of the processes taking place in plant syntheses. The present note concerns the search for protein in orange juice and some details of its characterization.

The fluid content of the orange as burred from the fruit consists of particles of so called pulp, suspended in the juice proper. The pulp in turn consists of the fibrous partitions of the pulp vesicles and the chromatophores contained in them. After centrifuging the crude juice, three layers are obtained: an upper layer consisting of the fibrous shreds; a middle fairly clear, light straw-colored fluid layer; and a bottom layer, deep orange in color and of gelatinous consistency. The top layer was skimmed off, whereupon the middle layer was poured off and filtered through paper

pulp. The clear juice contained about 12 per cent total solids and 0.1 per cent total nitrogen, of which one-third was amino nitrogen, as measured by the Van Slyke method. That the gaseous nitrogen liberated from this juice by nitrous acid does not arise entirely from amino acids seems likely,¹ though free amino acids have been isolated from alfalfa juice.² The very small relative increase in amino nitrogen after acid hydrolysis argues against the presence of an appreciable amount of protein in the clear juice. All attempts to demonstrate protein therein qualitatively resulted in failure, hence it is concluded that the clear juice contains no nitrogen in peptide combination.

Attention was then turned to the orange-red chromatophore material. After washing with water until free from acid it was extracted thoroughly for 24 hours with frequent shaking with 8 per cent sodium chloride solution. No protein was dissolved out by the water or by the saline solution. The material was then treated with 95 per cent alcohol. Although considerable oil and pigment was thus removed, no protein was detected in this extract. The residue was next treated with weak alkali (0.3 per cent NaOH). From the filtered alkaline extract 0.5 N acetic acid produced a yellow precipitate which flocked out and settled quickly at a pH of about 4.7. It was separated by centrifuging, and the pigment removed by repeated extractions with alcohol. The residue was finally washed with ether and dried in a current of warm air. After thorough extraction with aqueous soda solution, boiling with alkaline alcohol yielded no further protein.³

The grayish white powder obtained by the above treatment gives positive xanthoproteic, Millon's, Hopkins-Cole, and biuret tests. The color of the biuret test produced was almost pink, recalling that color observed with edestin and with proteose and peptone under similar conditions. However, when an alkaline solution of this protein was dialyzed for 46 hours against distilled water, no trace of protein was obtained in the dialysate, though

¹ Leavenworth, C. S., Wakeman, A. J., and Osborne, T. B., *J. Biol. Chem.*, 1923-24, lviii, 209.

² Vickery, H. B., *J. Biol. Chem.*, 1924, lx, 647; lxi, 117.

³ Osborne, T. B., Wakeman, A. J., and Leavenworth, C. S., *J. Biol. Chem.*, 1921, xlix, 63.

the alkali had passed through the membrane. The protein is insoluble in water, neutral salt solution, or weak acids, but is soluble in weak alkali. It is not precipitated from its solution by 95 per cent alcohol, nor is it coagulable by heat at neutral acid, or alkaline reactions.

A polysaccharide, resembling pectin, accompanies the protein fraction throughout the course of the extraction. It was found that repeated solution and reprecipitation of the protein reduced the amount of this carbohydrate obtained, though even after such treatment a positive Molisch and a qualitative test for pentose persisted.

Quantitative experiments showed that in the orange juice as burred, containing the orange-red bottom layer but not the fibrous shreds, there is at most 0.2 per cent protein, all of which is located in the highly pigmented material contained in the chromatophores of the pulp vesicles. Of the total nitrogen of this material about 10 per cent is extracted by strong alcohol and is presumably part of the pigment.

These experiments indicate that the protein found in the pulp of the orange is distinguished from any of the conventional classes of proteins by its unusual solubilities. Since the precipitation point of this protein is pH 4.7 and the pH of orange juice is 4.3, it follows that under ordinary conditions the protein is insoluble within the fruit. Although closely associated with the pigment, the protein is not apparently in chemical combination with it.

The author acknowledges the assistance of Mr. Harold Levine. Part of the expenses of this investigation was defrayed by the Research Laboratory of the California Fruit Growers Exchange.

A STUDY OF THE PHOSPHORUS, CALCIUM, AND ALKALINE RESERVE OF THE BLOOD SERA OF NORMAL AND RACHITIC CHICKS.*

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Drummond (1), Funk (2), Osborne and Mendel (3), and Hart, Halpin, and Steenbock (4, 5) have experimented with the baby chick as a laboratory animal with varying degrees of success in rearing it in confinement. The difficulty at once met with was that "leg weakness" frequently developed after 4 to 6 weeks in confinement. This disorder has been described from the standpoint of gross examination and observation of chicks under laboratory conditions. The work of Hart, Halpin, and Steenbock (4) led them to believe with Osborne and Mendel (3) that the failure was due to a lack of suitable roughage in the ration. They concluded that the disease was not a scurvy of chickens, since orange juice, green cabbage, or green clover failed to check the trouble. Their further efforts at rearing chickens in confinement were successful with the addition of cod liver oil to the ration.

Emmett and Peacock (6) concluded that chicks require the fat-soluble vitamin A while vitamin C was thought to be unnecessary. Hart, Steenbock, Lepkovsky, and Halpin (7) report results in agreement with those of Emmett and Peacock. Plimmer and Rosedale (8) believe that the addition of some foodstuff rich in vitamin B is advisable, while vitamin C does not appear necessary in the food of domestic and farm birds. Further, they state that the need of the chicken for vitamin B is greater than that of the pigeon. On the other hand, Sugiura and Benedict (9), working with pigeons, report that the fat-soluble vitamin is not essential in any stage of avian nutrition. They also found vitamin C unnecessary.

Herrick, Ackert, and Danheim (10) found light, roomy, well ventilated pens with clean wheat straw litter to be valuable assets in keeping chickens active and healthy.

Hart, Steenbock, Lepkovsky, and Halpin (11) studied the relation of light to the growth of the chicken, correlating the disturbance known as leg weakness with rickets. They found that "½ hour daily exposure to di-

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rect sunlight was much more potent in furnishing the antirachitic equivalent than was 5 per cent of a synthetic ration fed as fresh green clover, calculated on the basis of the dry weight of the clover." Their findings were substantiated by work reported by Hughes (12). They furthermore showed (13) that cod liver oil, freed from vitamin A by aeration was efficacious in relieving the symptoms of leg weakness in chickens. They argued that this fact furnished additional justification for believing the antirachitic vitamin to be distinct from vitamin A.

Dunn (14), working with single combed White Leghorn chicks, found that cod liver oil fed as 0.5 per cent of a ration free from the fat-soluble and the antirachitic substances, but complete in other respects, is sufficient to prevent rickets in chickens reared in strict confinement indoors. This work has been substantiated by evidence gathered by Mussehl¹ which showed that either cod liver oil or direct sunlight, added to a diet otherwise complete save for the antirachitic factor, will induce normal growth and freedom from leg weakness.

Hart, Halpin, and Steenbock (5) determined the inorganic phosphorus of the blood serum of chicks fed their basal diet of white corn, 97 parts; calcium carbonate, 2 parts; sodium chloride, 1 part; and skimmed milk *ad libitum*, with and without the addition of cod liver oil. They reported a lower content of inorganic phosphorus in the blood serum of the group from which cod liver oil was withheld. Later work (13) from their laboratory demonstrated that both the phosphorus and the calcium of the blood serum were restored to normal by the addition of cod liver oil freed from vitamin A by aeration.

The work of Mussehl¹ gave us an excellent opportunity to study the effect of a rachitic diet on the calcium and inorganic phosphorus content of the blood serum of growing chicks. The basal ration used by Steenbock was admittedly incomplete, whereas the basal ration used in this work was more complex, and naturally more complete in its dietary requirements. Furthermore, we were able to use more individuals since each lot was made up of thirty-five birds. The work included, in addition to cod liver oil treatment, the prevention of rickets by direct sunshine. The changes taking place in rickets were also studied under four different conditions. The purpose of the investigation was to determine whether the changes found by Steenbock on addition of cod liver oil to their ration would appear with a more complete ration, and also to note if the same changes accompanied the prevention of rickets by direct sunshine.

EXPERIMENTAL.

The chicks used in the work herein reported were single combed White Leghorns, incubator-hatched, selected at an age of from 7 to 10 days, at which age it was possible to discard all abnormal

¹ Mussehl, F. E., unpublished data.

chicks. They were then placed in lots of thirty-five chicks on the following basal ration, designated as N. E.

Scratch feed.	<i>parts</i>
Cracked wheat.....	50
“ yellow corn.....	50

This was fed in clean shavings.

Mash.	<i>per cent</i>
Yellow corn-meal.....	21.25
Wheat bran.....	21.25
“ shorts.....	21.25
Pulverized barley.....	21.25
Meat meal.....	15.00

The different components of the mash were finely ground and intimately mixed so that selection was impossible. The mash was fed in metal hoppers. The feeding practice was to balance equal quantities of mash and scratch feed. Sprouted oats were furnished for succulence to all lots.

The brooding conditions were excellent as shown by the very low mortality during the first 3 weeks, or until deficiencies in the diet began to show up. Electrically heated hovers were used.

To furnish a control group, three lots were placed on the N. E. ration and received their sunlight filtered through ordinary window glass. The failure of these chicks to grow normally, and the incidence of leg weakness, or rickets, is shown by Curve E of Chart 1.

That the N. E. ration is complete in all its dietary requirements save for the antirachitic factor is evidenced by the fact that when four lots were placed on it, but in addition were exposed to the direct rays of the sun, normal growth was obtained. These results are shown by Curve A. The incidence of a few cases of rickets in one of these lots (Curve C) may be explained by the lack of sunshine at that season of the year, and the cloudy weather encountered.

A similar group of four lots was placed on the N. E. ration plus 1.5 per cent cod liver oil, but not exposed to direct sunlight. Normal growth, comparable to the group in direct sunlight, was obtained uniformly. This is in accord with the generally accepted theory that sunlight and cod liver oil replace each other as a preventative of rickets. Curve B illustrates this.

One lot of birds was given ration N. E. plus direct sunlight and 5 per cent of dried yeast. That the basic ration is complete with respect to the water-soluble vitamin is shown by the fact that no better growth was obtained in this group than in the direct sunlight group. Curve H shows the results of this trial.

A copper arc light was used with two lots in an attempt to prevent the occurrence of rickets, but both attempts resulted in failure as judged from the rickets present in the lots and the poor growth coupled with high mortality. This can be seen from a consideration of Curve F.

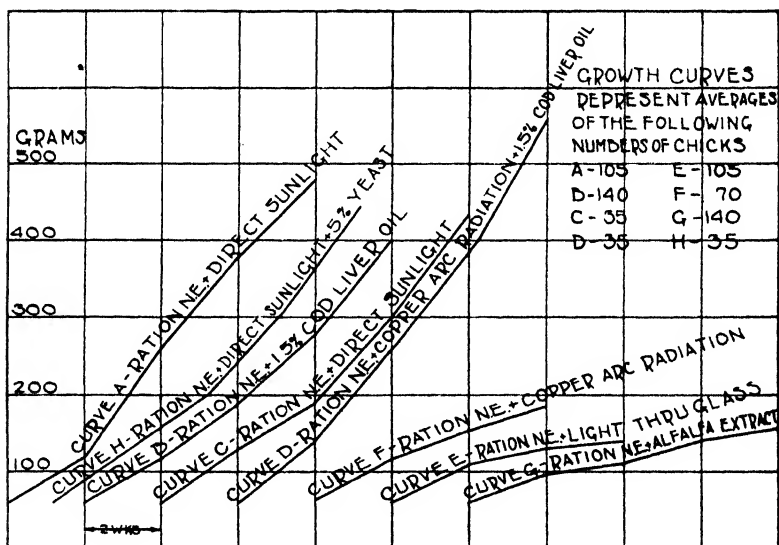


CHART 1.

In order to determine whether copper arc light radiation increased the efficiency of the cod liver oil addition to the N. E. ration, one lot was tested under these conditions. Curve D shows that normal growth was secured, and that no distinct advantage resulted from the radiation.

Shipley, Kinney, and McCollum (15), working with rats, state that extracts of alfalfa meal made with boiling water are antirachitic. In order to determine whether this was true with chicks, one lot had the water extract of 250 gm. of well cured, green al-

alfa meal added to each kilo of the basic ration, and another, the water extract of 500 gm. When these trials resulted in the usual symptoms of rickets, two other lots were placed on the basic ration, to which was added the liquid pressed out of fresh, green alfalfa with a hydraulic press at a pressure of 4,000 pounds per square inch. One of these lots received the hydraulic extract of 225 gm. of fresh, green alfalfa per kilo of basal ration, and the other lot got the extract of 450 gm. Contrary to the findings of Shipley, Kinney, and McCollum (15) with rats, both of these lots resulted in failure to prevent rickets in chicks, as shown by Curve G.

Poor growth and a high mortality resulted from the attempt to furnish the antirachitic factor by the water extract of either cured alfalfa or the expressed juice of fresh, green alfalfa. The fact that doubling the quantity of extract given produced no better results shows that the fault did not lie in the amount given. Furthermore, as noted in Table I, the concentrations of calcium and the inorganic phosphorus of the blood serum place the lots in the group of rachitic birds, thus confirming the incidence of rickets.

In order to determine the effect of the withdrawal of the antirachitic equivalent from the lot on 1.5 per cent cod liver oil, six cockerels with an average weight of 419 gm. were continued on the N. E. ration without direct sunlight or cod liver oil. In 10 days these birds showed the characteristic leg weakness induced by a faulty ration. Their rapid decline was doubtless due to the fact that there had been no opportunity for storage of any protective factor.

For the determination of phosphorus the Briggs (16) modification of the Bell-Doisy method was used in the earlier work. His later changes (17) were adopted shortly after they appeared. A Bock-Benedict colorimeter was employed in making the color comparisons. This was set at 20 mm. Calcium was determined by the method of Kramer and Tisdall (18) in all analyses. The plasma bicarbonate was determined by Van Slyke's method (19).

The chicks were kept on the various rations until blood samples were desired. They were then taken from the lot and blood samples drawn from the right auricle by means of a 10 cc. hypodermic syringe. (That the right auricle was invariably entered was confirmed by a

postmortem examination of several individuals.) The size of the needle used was varied to correspond to the size of the bird. Sodium citrate was used as an anticoagulant, the practice being to coat the inside of the syringe and centrifuge tubes, which was found effective. In drawing blood for the bicarbonate determination, mineral oil was used at every stage to prevent the access of air to the drawn blood.

Blood was taken directly from the heart to avoid the possibility of contamination accompanying the severing of the neck and collection of the blood in tubes. Stasis is likewise prevented by this method as compared with drawing blood from the brachial vein. It permits greater economy of the blood, since there is no loss. No anesthesia was used, the heart being quickly entered and the desired amount of blood withdrawn. The sample obtained was then centrifuged at high speed, following which operation the serum was poured off in order to prevent it from standing in contact with the clot. The desired amount of serum was then taken for the various determinations, which were made immediately.

One advantage of this method is that the bird need not be sacrificed to obtain a sample of blood, as there seems to be very little shock. In a very few minutes the chicks are on their feet, and in 2 or 3 days it is impossible to pick them out of the lot. An amount of blood equal to 6 per cent of the body weight was taken from one individual with no lasting detrimental result. While this method of drawing blood is a common practice with guinea pigs and other laboratory animals, we have seen no reference to its use with chicks.

DISCUSSION.

It will be seen from a consideration of Table I that the groups naturally fall into two divisions as regards the amount of calcium and inorganic phosphorus in the blood serum. The lots receiving cod liver oil additions or direct sunshine were uniformly free from rickets, and were certainly normal as far as growth was concerned. Their sera have slightly higher contents of calcium and inorganic phosphorus than those where the antirachitic factor was withheld. Thus, in a gross comparison, 56 birds in the cod liver oil-arc light, the direct sunshine, and cod liver oil groups, have an average calcium content of 10.61 mg. per 100 cc. of blood serum.

In like manner, 68 birds from these same groups, in most of the cases from the same birds, have an average content of 4.60 mg. of inorganic phosphorus per 100 cc. of blood serum. This latter figure is in agreement with the figure reported on three controls by Steenbock and coworkers (13).

TABLE I.

	Cod liver oil- are light.	Sunshine.	* Cod liver oil.	Big rickets.	Are light.	Check.	Extract alfalfa.
Average calcium*	11.73	10.04	9.64	7.72	7.35	7.17	7.16
Maximum "	14.85	12.53	11.93	9.41	9.03	9.03	9.75
Minimum "	9.80	6.12	8.32	5.34	6.50	4.97	4.97
No. of individuals	16	23	13	4			1
Composites†	1-4				6-15	6-19	5-12
Average phosphorus*	4.21	4.77	4.59	3.92	3.73	3.99	3.82
Maximum "	6.29	6.89	5.69	4.76	4.92	4.90	4.99
Minimum "	3.8	3.00	3.57	3.15	3.10	3.48	2.95
No. of individuals	9	30	20	6			1
Composites†	3-9				11-25	7-22	5-12
Plasma bicarbonate	52.2	55.9	55.2	52.1	56.0		
Maximum CO ₂	57.8	62.7	61.6	56.4	56.0		
Minimum CO ₂	47.5	47.0	51.1	50.4	56.0		
No. of individuals	10	17	12	6	2		
Average P × Ca	49.4	47.9	44.2	30.2	27.4	28.7	27.4
Maximum P × Ca	93.4	74.9	54.0	42.2	36.7	31.4	38.7
Minimum P × Ca	38.6	33.5	31.0	23.2	25.7	20.2	14.7

* Expressed in terms of mg. of calcium and inorganic phosphorus per 100 cc. of blood serum.

† Indicates the number of pooled samples of serum, and the number of birds in the pools. Thus, 6-15 means there were 6 pooled samples, therefore six determinations, and the total number of birds in the six pools was 15.

In the other groups, where rickets regularly appeared, 51 chicks had an average content of 7.49 mg. of calcium per 100 cc. of blood serum. From this same group, in most instances the same birds, 66 chicks gave an average value of 3.91 mg. of inorganic phosphorus per 100 cc. of blood serum.

The group with the cod liver oil-are light additions had the highest content of calcium, while the other groups of rickets-free birds had a slightly lower figure. With respect to the inorganic phosphorus there was no significant variation. In the four groups where rickets developed, the figures for both the calcium and phosphorus were quite constant.

There were no significant changes in the volume per cent of plasma bicarbonate, either as between rachitic and non-rachitic birds, or between the several lots. The figures obtained fall at the lower end of the range obtained by Van Slyke (20) for normal resting adults. Schloss and Stetson (21) reported a lower value for normal infants than for adults. Since the figures herein reported are for young chickens, the same variation may be present in chickens as in man.

Howland and Kramer (22) and Cavins (23) agree that "there is a close relationship between the deposition of calcium phosphate in bone and the product of the concentration of the serum calcium (in mg. per 100 cc.) into the concentration of inorganic phosphorus, expressed in the same terms" and that "when the product is below 30, rickets is to be expected, between 30 and 40 it is probable. When the product is above 40 either healing is taking place or rickets is entirely absent." That this statement holds true with chicks is shown conclusively in Table I.

We failed to obtain the increase of sera phosphorus or calcium noted by Steenbock and coworkers (13) upon addition of cod liver oil to the ration. That the cod liver oil used was efficacious in preventing rickets is shown by the failure of the check lots as compared with the lots receiving the cod liver oil addition with neither direct sunshine nor copper arc light. The difference apparently lies in the nature of the treatment given. Steenbock's chickens were transferred from the standard poultry ration to the rickets-producing ration mentioned above. Then, when failure set in, cod liver oil was added to the ration. At this Station, the comparison was made between two lots, one receiving from the beginning the N. E. ration plus cod liver oil, and the other the N. E. ration alone.

SUMMARY.

The average content of inorganic phosphorus of the blood serum of 68 normal, growing, single combed White Leghorn chicks was

found to be 4.60 mg. per 100 cc. of blood serum, and the calcium content of the serum of 56 birds was found to be 10.61 mg.

In 66 chicks suffering from rickets, the inorganic phosphorus of 100 cc. of blood serum averaged 3.91 mg. and the calcium content of the serum of 51 chicks, 7.49 mg.

No significant changes occurred in the plasma bicarbonate content of the blood serum of rachitic chicks as compared with normal ones.

The theory of Howland and Kramer, that when the product of the concentration of the inorganic phosphorus into the calcium, expressed in terms of milligrams per 100 cc. of blood serum, is above 40, rickets is either absent or healing, is confirmed for chicks.

Neither the water extract nor the expressed juice of fresh green alfalfa prevented the onset of rickets on a diet complete save for the antirachitic factor. This is in disagreement with the findings of McCollum with rats.

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OXIDATION OF *d*-2-MERCAPTOBUTANE TO *d*-BUTANE-2-SULFONIC ACID, AND THE ROTATIONS OF THE SALTS AND FREE ACIDS OF THE THIO- AND SULFOCARBOXYLIC ACIDS.

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Previous publications of this series contained reports dealing with the effect on the optical rotation of the change in the polarity of one of the groups attached to the asymmetric carbon atom. The following substances have been investigated in this direction: *l*-2-mercapto-octane, *d*- α -thiolactic acid, and *d*-thiosuccinic acid. In the present paper is discussed the change in optical rotation which is connected with the conversion of *d*-2-mercaptobutane into the corresponding 2-butanedisulfonic acid. As in the case of the derivative of octane, so in that of butane, the conversion of the mercaptane into the sulfonic acid is accompanied by a change in direction of rotation. The former has a molecular rotation of $+14.13^\circ$, the latter of -4.38° .

Table I contains the results of the observations made up to the present date.

The analysis of the results of the observations given in Table I shows that in the derivatives of the alcohols, there is a definite reversal of the direction of rotation. The quantitative differences in rotation cannot as yet be ascertained, owing to the circumstance that every step in the preparation of mercaptane and of the sulfonic acids may have been accompanied with a certain degree of racemization.

In the derivatives of the substituted acids no change in the direction of rotation was observed. Again in this series, the preparation of the substances is accompanied with a certain degree of racemization and one cannot know definitely whether a change in the magnitude of rotation does occur when a thio

acid is converted into the sulfonic acid. If the drop in dextro-rotation which is noted in Table II be real, then it may be considered that the change of the SH group into that of SO_2OH brings about in the derivatives of the alcohols and in those of α -hydroxy acids qualitatively the same character of change of rotation. This problem can be solved more accurately by resolving the partially racemized substances into the pure forms of the thio and of the sulfonic acids. It is intended eventually to accomplish this task.

TABLE I.
Optical Activity of Thio and Sulfo Acids.

Starting material	$[\text{M}]_D$ of SH derivative.	$[\text{M}]_D$ of sulfonic acid.
<i>l</i> -Hexylmethyl carbinol.	-11.75°	+4.92°
<i>d</i> -2-Butanol.	+14.13°	-4.38°
<i>d</i> -Propionic acid.	+58.98°	+13.76°
Malic acid	+73.06°	+44.25°

TABLE II.
Optical Activity of the Salts of Thio and Sulfo Acids.

Substance.	Free acid. $[\text{M}]_D$	Mono-salt. $[\text{M}]_D$	Di-salt. $[\text{M}]_D$	Tri-salt. $[\text{M}]_D$
<i>l</i> -Xanthopropionic acid.	+107.68°	+45.05°		
<i>l</i> -Thiolactic acid.	+58.98°	-5.58°	+7.32°	
<i>l</i> -Sulfopropionic acid.	+13.76°	+12.84°	-3.28°	
<i>l</i> -Xanthosuccinic acid.	+95.05°	+43.86°	+8.65°	
<i>l</i> -Thiosuccinic acid.	+73.06°	+38.04°	+48.57°	+41.09°
<i>l</i> -Sulfosuccinic acid.	+51.53°	+49.27°	+37.89°	+25.67°
<i>d</i> -Sulfobutane.	-4.38°	-6.29°		

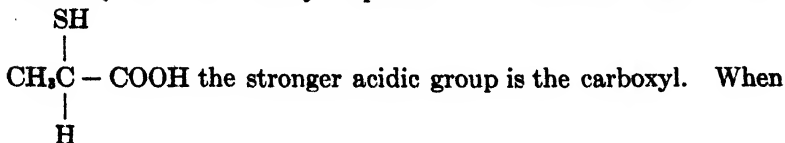
However, the principal object of the present work is to find a way to establish configuration relationships between optically active substances differing in only one respect; namely, in the polarity of one group attached to the asymmetric carbon atom.

In the series of carbinols and simple halides the problem is somewhat less complicated, particularly when the substances are liquids and the rotations of the pure homogeneous substances can be compared. The problem is more complicated in the series of substituted carboxylic acids. In the latter, the salts

and the corresponding acids frequently rotate in opposite directions. Thus, the salts of *d*-lactic acids rotate to the left. From the article which follows¹ it is seen that in sugar acids in which the carbon atom 2 has the same configuration as in *d*-gluconic acid, the salts have a higher dextrorotation than the free acids. In this respect levo- α -hydroxy acids behave as *d*-gluconic acid.

If one now compares the rotation of a *d*-mercaptane and the corresponding sulfonic acids, he finds that the direction of the rotation of the latter is reversed, and, furthermore, that its salt has a higher levorotation than the free acid. One may say, therefore, that this acid behaves like an α -hydroxycarboxylic acid of the *l* series, although it has the configuration of a dextrorotatory mercaptane. Similarly, the conversion of the active *d*-amyl alcohol into the corresponding valeric acid is accompanied by a reversal of the direction of rotation, and the salt has a higher molecular rotation to the left than the acid. Thus, in this series of substances, the conversion of an alcoholic group to a carboxyl or of a thio group to a sulfonic leads to a reversal of the direction of the rotation, and, furthermore, the resulting acids have a lower levorotation than their salts, in this respect behaving similarly to the hydroxy acids of the *l* series.

In the α substituted acids the transformation of the thio acids into the corresponding sulfonic acids brings about optical changes which at first seem more difficult to explain. Thus dextrothiolactic acid forms a levorotatory monobasic salt. It behaves in this respect similarly to dextrolactic acid. The direction of rotation, however, is again changed when a second equivalent of sodium hydroxide is added to the solution. Thus the neutral salt is dextrorotatory. In the sulfopropionic acid the direction of rotation is unaltered when the acid is converted into a monobasic salt, and is reversed in the dibasic salt. This apparently puzzling difference in the behavior of the two substances, however, can be readily explained. In dextrothiolactic acid



¹ Levene, P. A., *J. Biol. Chem.*, 1925, lxxiii, 95.

the hydrogen of this group is replaced by a metal ion, the substance behaves as a hydroxy acid of the *l* series; namely, the salt is less dextrorotatory than the free acid. When a second equivalent of the base is added, it combines with the sulfide rest and then the direction of the rotation turns to the right, as in α -hydroxy acids of the *d* series.

OH

|

SO₂

|

In the sulfopropionic acid CH₃—C—COOH the stronger

|

H

acidic group is the rest SO₂OH. The neutralization of this group brings about a slight change in the direction toward the left, as in α -hydroxy acids of the *l* series. When a second equivalent of base is added, this forms a salt with the carboxyl group and then the direction of the rotation is shifted to the left as in the α -hydroxy acids of the *l* series.

One can arrive at a similar conclusion from the observations of Backer and de Boer.² These authors compared the rotations of sulfobutyric acid with those of the neutral salt of the acid and observed that in the salt the direction of the rotation was reversed, but when the anilid of the acid was prepared, the salt and the free acid rotated in the same direction.

With respect to the carboxyl, then, the thio and the sulfo acids belong to the same series. Thus, accepting the rule that to the *d* series belong such α -hydroxy acids in which the difference of the value of the molecular rotations of the salt and its free acid is positive, and to the *l* series those in which this is negative, we shall conclude that if the polarity of one of the groups in the α substituted acids is changed, the new acid still behaves as an acid of that series (*d* or *l*) to which the original acid belonged.

On the other hand, the substitution by a metal ion of the hydrogen atom of the sulfonic group has an opposite effect from that of the thio group.

The same regularity obtains in the derivatives of succinic

² Backer, H. J., and de Boer, J. H., *Rev. trav. chim. Pays-Bas*, 1924, xliii, series 4, 297, 420.

acid. Xanthosuccinic acid contains two oxidic groups, both carboxylic. The neutralization of each is accompanied with a drop in dextrorotation. The thiosuccinic acid contains three acidic groups. The first and the third act as in xanthosuccinic acid and hence are the carboxyl groups. The second acidic group, on neutralization, gains in dextrorotation and, therefore, may be assumed to be the thio group. By analogy with substituted propionic acids, one should expect that when the thio group is oxidized to the sulfonic group the neutralization of this group should cause a drop in dextrorotation. The sulfonic acid group is readily recognized by being the strongest acidic group. Hence it is expected that the monobasic salt of sulfopropionic acid should have a lower dextrorotation than the free acid. By analogy, the neutralization of each of the carboxyls should also lead to a drop in dextrorotation, and this drop was actually observed.

Thus, also, for the substituted succinic acids, the rule holds that the change in polarity of the substituting group does not affect the character of change in rotation brought about by neutralization of the carboxylic group.*

EXPERIMENTAL.

d-2-Mercaptobutane.—The iodobutane used in this experiment was prepared according to the directions of Pickard and Kenyon.³ 20 gm. of *d*-β-iodobutane were heated with $1\frac{1}{4}$ mols of alcoholic potassium hydrogen sulfide solution. The mixture was heated for 2 hours under a return condenser and was then poured into water. The mercaptane was drained off by means of a separatory funnel. The product weighed 6 gm. and was found to be free from combined iodine. This product was combined with the mercaptane obtained in previous experiments and subjected to fractional distillation. A little of the substance distilled over under 85°C., whereas the bulk went over at 85–95°C. The first fraction was discarded. The optical activity of the mercaptane was determined in a $\frac{1}{2}$ dm. tube without solvent. The reading was found to be + 6.52°. The specific gravity of the mercaptane is 0.8299.

* Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 64.

$$[\alpha]_D^{20} = \frac{+6.52^\circ}{0.5 \times 0.8299} = +15.71^\circ. \quad [M]_D = +14.13^\circ.$$

0.2036 gm. substance: 0.5232 gm. BaSO₄.

C₄H₁₀S. Calculated. S 35.61.

Found. " 35.30.

d-Butane-2-Sulfonic Acid.—5 gm. of *d*-butyl mercaptane were dropped slowly into a mixture of 10 cc. of concentrated nitric acid and 2 cc. of water. The flask with the nitric acid was fitted with a return condenser, while the mercaptane was added from a dropping funnel. The reaction was very vigorous. If the mercaptane is added too fast spontaneous combustion of the mercaptane takes place in the fumes of the nitric acid. The mixture was heated under a return condenser for 1½ hours, whereupon it was transferred to a casserole and the heating continued for another ½ hour. It was then transferred into a distilling flask and the excess of nitric acid was removed under reduced pressure. The residue was taken up with water and treated with excess of barium carbonate. The excess of barium carbonate were filtered off, and the filtrate concentrated and subsequently treated with alcohol. The salt was precipitated in crystalline form. The dissolution in water and precipitation with alcohol were repeated. Two fractions were obtained. 1.3368 gm. of the first fraction were dissolved in water and diluted to 10 cc. Reading was taken in a 1 dm. tube.

$$[\alpha]_D^{20} = \frac{-0.41^\circ \times 100}{1 \times 13.368} = -3.06^\circ. \quad [M]_D = -6.29^\circ.$$

1 cc. of concentrated hydrochloric acid was then added, thus bringing the volume to 11 cc. Rotation was again determined in a 1 dm. tube.

$$[\alpha]_D^{20} = \frac{-0.26^\circ \times 100}{1 \times 8.161} = -3.18^\circ. \quad [M]_D = -4.38^\circ.$$

0.1910 gm. substance: 0.2176 gm. BaSO₄.

0.0955 " " : 0.0532 " "

C₆H₅O₁₀S₂Ba. Calculated. S 15.59, Ba 33.30.

Found. " 15.69, " 32.78.

d-Sodium Thiolactate.—3 gm. of *d*-thiolactic acid, $[M]_D = +58.98^\circ$, were dissolved in water and treated with 1 equivalent

of sodium carbonate. The solution was then diluted to 10 cc. and rotation determined in a 1 dm. tube.

$$[\alpha]_D^{25} = \frac{-1.58^\circ \times 100}{1 \times 36.22} = -4.36^\circ. \quad [M]_D = -5.58^\circ.$$

The above solution was treated with 1 equivalent of sodium hydroxide. The total volume was now 13.5 cc.

$$[\alpha]_D^{25} = \frac{+2.12^\circ \times 100}{1 \times 43.39} = +4.88^\circ. \quad [M]_D = +7.32^\circ.$$

d-Sodium- α -Sulfopropionate.—2.81 gm. of *d*-barium- α -sulfo-propionate were converted into the sodium salt by treating the former with 1 mol of sodium sulfate in aqueous solution. The mixture was shaken for 1 hour at room temperature and then filtered. The precipitate was washed with water. The combined filtrates amounted to 12 cc. Rotation was determined in a 1 dm. tube.

$$[\alpha]_D^{25} = \frac{-0.32^\circ \times 100}{1 \times 19.23} = -1.66^\circ. \quad [M]_D = -3.28^\circ.$$

1 equivalent of hydrochloric acid was now added. The total volume was now 12.6 cc.

$$[\alpha]_D^{25} = \frac{+1.02^\circ \times 100}{1 \times 13.97} = +7.30^\circ. \quad [M]_D = +12.84^\circ.$$

1 more equivalent of HCl was added. Volume = 15.2 cc.

$$[\alpha]_D^{25} = \frac{+0.88^\circ \times 100}{1 \times 9.842} = +8.94^\circ. \quad [M]_D = +13.76^\circ.$$

d-Sodium Xanthopropionate.—1.253 gm. of *d*-xanthopropionic acid were treated with 1 equivalent of sodium hydroxide and then diluted to 10 cc. Rotation was determined in a 1 dm. tube. The weight of the acid taken corresponds to 1.395 gm. of the monosodium salt.

$$[\alpha]_D^{25} = \frac{+2.91^\circ \times 100}{1 \times 13.95} = +20.86^\circ. \quad [M]_D = +45.05^\circ.$$

The free acid showed a rotation of

$$[\alpha]_D^{25} = \frac{+2.81^\circ \times 100}{1 \times 5.062} = +55.51^\circ. \quad [M]_D = +107.68^\circ.$$

d-Monosodium Xanthosuccinate.—The activity of *d*-xanthosuccinic acid was determined in aqueous solution in a 1 dm. tube.

$$[\alpha]_D^{25} = \frac{+2.02^\circ \times 100}{1 \times 5.189} = +38.92^\circ. \quad [M]_D = +95.05^\circ.$$

0.5264 gm. of the above acid was treated with 1 equivalent of sodium hydroxide and the volume made up to 10 cc. The acid taken corresponds to 0.5750 gm. of the monosodium salt.

$$[\alpha]_D^{25} = \frac{+0.97^\circ \times 100}{1 \times 5.75} = +16.87^\circ. \quad [M]_D = +43.86^\circ.$$

In another experiment 0.3846 gm. of the acid was treated with 2 equivalents of sodium hydroxide and then diluted to 10 cc. The acid corresponds to 0.4557 gm. of the disodium salt.

$$[\alpha]_D^{25} = \frac{+0.14^\circ \times 100}{1 \times 4.557} = +3.07^\circ. \quad [M]_D = +8.65^\circ.$$

d-Mono-, Di-, and Trisodium Thiosuccinates.—Activity of the free acid was determined in aqueous solution.

$$[\alpha]_D^{25} = \frac{+5.62^\circ \times 100}{1 \times 11.537} = +48.71^\circ. \quad [M]_D = +73.06^\circ.$$

To determine the optical activity of the monosodium salt 1.2220 gm. of the acid were treated with 1 equivalent of sodium hydroxide and the volume made up to 10 cc. The acid corresponds to 1.5315 gm. of the monosodium salt.

$$[\alpha]_D^{25} = \frac{+3.39^\circ \times 100}{1 \times 15.315} = +22.12^\circ. \quad [M]_D = +38.04^\circ.$$

In a similar manner the activity of the di- and trisodium salts was determined by treating the acid with 2 and 3 equivalents of sodium hydroxide, respectively.

$$\text{For monosodium salt } [\alpha]_D^{25} = \frac{+3.39^\circ \times 100}{1 \times 15.315} = +22.12^\circ. \quad [M]_D = +38.04^\circ.$$

$$\text{For disodium salt } [\alpha]_D^{25} = \frac{+3.30^\circ \times 100}{1 \times 13.171} = +25.04^\circ. \quad [M]_D = +48.57^\circ.$$

$$\text{For trisodium salt } [\alpha]_D^{25} = \frac{+2.56^\circ \times 100}{1 \times 13.129} = +19.49^\circ. \quad [M]_D = +41.08^\circ.$$

d-Tri-, Di-, and Monopotassium Sulfosuccinates.—0.3996 gm. of the dipotassium salt was treated with 1 equivalent of potassium hydroxide and the volume made up to 10 cc. This corresponds to 0.4269 gm. of the tripotassium salt.

$$[\alpha]_D^{25} = \frac{+0.35^\circ \times 100}{1 \times 4.269} = +8.19^\circ. \quad [M]_D = +25.67^\circ.$$

0.7216 gm. of the dipotassium salt was dissolved in water and the volume made up to 10 cc.

$$\text{For the anhydrous salt } [\alpha]_D^{25} = \frac{+0.93^\circ \times 100}{1 \times 6.724} = +13.83^\circ. \quad [M]_D = +37.89^\circ.$$

The activity of the monopotassium salt and of the free acid was obtained by treating a solution of the di-salt with 1 and 2 equivalents of hydrochloric acid, respectively.

$$[\alpha]_D^{25} = \frac{+1.12^\circ \times 100}{1 \times 5.363} = +20.88^\circ. \quad [M]_D = +49.27^\circ.$$

$$[\alpha]_D^{25} = \frac{+1.08^\circ \times 100}{1 \times 4.148} = +26.03^\circ. \quad [M]_D = +51.53^\circ.$$

THE CONFIGURATION OF 2-AMINOHEXONIC ACIDS AND OF 2-AMINOHEXOSES.

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Many efforts have been made to correlate the configuration of amino acids with that of the halogen acids and with that of the hydroxy acids (particularly of the α substituted series). The writer was particularly interested in that phase of the problem which was concerned with the configuration of the amino sugars and the amino sugar acids. Because of the phenomenon of Walden inversion the problem has to be solved by indirect methods involving a consideration of the physical properties of the substances and of their derivatives. One of the principal difficulties lay in the fact that the direction of rotation of a given substance was not constant, but varied with the variation of external conditions. Thus, the rotation of hydroxy acids frequently varies with the change of concentration, with the change of temperature, with the change of the solvent, etc. It was the convention to name all such acids as *d* acids, the salts of which had a dextro-rotation, and all such α -amino acids as *d* acids, which had a dextrorotation in a solution of a mineral acid. It was realized, however, that a classification based on these assumptions was unsatisfactory, and several authors, particularly Hudson, Clough, Freudenberg, and the writer, were engaged in finding more reliable criteria for classifying a substance into the *d* or *l* series. Since the conclusions are to be based on analogies of behavior, the writer thought of selecting a group of substances which permitted the verification of a given conclusion by the behavior of a considerable number of derivatives. From this view-point the amino sugars and the corresponding sugar acids represent the most satisfactory material.

In previous discussions of the relationship of hexonic and 2-

TABLE I.

Acid.	$[\alpha]_D$ of carbon atom 2.	$[M]_D^{25}$	Phenyl- hydrazide.	$[\alpha]_D$ of carbon atom 2.	$[M]_D^{25}$
Epichitosaminic.....	+12.5	+24.37 (10 ²)	Gluconic.....	+14.25	+42.18 (10 ²)
Chitosaminic.....	-12.5	-24.37 (10 ²)	Mannonic.....	-14.25	-42.18 (10 ²)
Dextroxylohexosaminic.....	+12.5	+24.37 (10 ²)	Gulonic.....	+14.25	+42.18 (10 ²)
Levoxylohexosaminic.....	-12.5	-24.37 (10 ²)	Idonic.....	-14.25	-42.18 (10 ²)
Epichondrosaminic.....	+12.5	+24.37 (10 ²)	Galactonic.....	+8.25	+24.42 (10 ²)
Chondrosaminic.....	-12.5	-24.37 (10 ²)	Talonic.....	-8.25	-24.42 (10 ²)
Dextroribohexosaminic.....	+19.12	+37.28 (10 ²)	Allonic.....	+20.8	+61.56 (10 ²)
Levoribohexosaminic.....	-19.12	-37.28 (10 ²)	Altronic.....	-20.8	-61.56 (10 ²)

aminohexonic acids the writer was greatly influenced by the convention of comparing the optical behavior of the salts, or still better, the hydrazides of the hexonic acids with those of the hydrochlorides of the 2-aminohexonic acids.

It was found that the specific rotation of carbon atom 2 had closely approaching values only when the hydrazides of the hexonic acids and the hydrochlorides of the 2-aminohexonic acids were taken as the bases for calculation. When the free acids of the two series were compared, no such regularity was found. On the basis of this comparison, the relationships are given in Table I. These conclusions seemed to be substantiated by the equilibria which were established between the two epimers which are obtained on the addition of prussic acid to pentoses, on one hand, and to the aminopentoses, on the other. Furthermore, this assumption was substantiated by Fischer's view on Walden inversion according to which a dextro- α -amino acid on deamination with nitrous acid yielded a levo- α -hydroxy acid.¹

On the basis of these considerations, the configuration of 2-aminomannonic acid should be assigned to chitosaminic acid and that of 2-aminomannose to chitosamine.

On the other hand, if an attempt is made to base a theory of the structure of chitosamine on the properties of the two respective pairs of epimers, glucose and mannose, on the one hand, and chitose and epichitose on the other, a directly opposite conclusion is reached. Thus it was pointed out by Irvine that the difference between the rotations of the α - and β -chitosamine hydrochlorides is normal, as in glucose, and, therefore, he attributed to chitosamine the configuration of 2-aminoglucose. This argument alone is scarcely sufficient to establish the configuration of the amino sugar inasmuch as the difference in rotation of the α - and β -isomers is a function of the carbon atoms 1 and the behavior of these may be influenced differently by the proximity of an amino group than by that of a hydroxyl. Irvine's argument,

¹ It is suggested to name as dextro all such acids which rotate to the right in the free state and as *d* acids those acids the salts of which have a higher dextrorotation than the free acids. Thus levulactic acid (levorotatory lactic acid) is a *d* acid inasmuch as its salts have a higher dextrorotation than the acid and dextro-sulfopropionic acid (dextrorotatory) is an *l* acid because the neutral salt has a lower dextrorotation than the acid.

98 2-Aminohexonic Acids and 2-Aminohexoses

however, gains in importance when the behavior of epichitosamine also is taken into consideration, for, similarly to the case of mannose, the difference in the rotations of the α - and β -isomers of epichitosamine hydrochloride is abnormal. Furthermore, if the direction of rotation of carbon atom 2 in glucose and in mannose is determined on the basis of the molecular rotations of the α - and β -isomers of glucose and of those of α - and β -isomers of mannose, it is found that in glucose it rotates to the right and in mannose to the left. The same conclusion is reached when the rotations of the pentacetates are employed instead of the simple

TABLE II.

Showing the Specific Rotations of the Free Acids and of Their Derivatives (All of the d Series).

	Free acids.* [α] _D ²⁰	Na salts.† [α] _D ²⁰	Phenylhy- drasides.‡ [α] _D ²⁰	Amides.§ [α]
Gluconic.....	0.0	+11.78	+18.0	+31.2
Mannonic.....	+15.6	-8.82	-10.5	-17.3
Idonic.....	+	-2.52	-15.1	
Gulonic.....	-1.6	+12.68	+13.45	+15.2
Galactonic.....	-8.0	+0.40	+12.2	+30.0
Talonic.....	?	?	+4.35	
Allonic.....	-10.0	+4.30	+25.88	
Altronic.....	+8.0	-4.05	-15.8	

* Recent observation.

† Observations by Levene and Meyer (Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1916, xxvi, 365).

‡ Observations by Levene and Meyer (Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 625).

§ Observations by Hudson and Komatsu (Hudson, C. S., and Komatsu, S., *J. Am. Chem. Soc.*, 1919, xvi, 1142).

sugars. When the pentacetates of chitosamine and epichitosamine are compared in the same respect, it is found that the carbon atom 2 rotates to the right in chitosamine (as in glucose) and to the left in epichitosamine (as in mannose).

Thus, the conclusions reached on the basis of the optical behavior of the sugars seem to be contradictory to those observed on the 2-aminohexonic acids.

A uniform conclusion can be reached from the optical behavior of the two sets of substances if the classification of α substituted

TABLE III.

Acids.	In 5 per cent NaOH.	In 2.5 per cent NaOH.
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On page 99, Vol. lxiii, No. 1, February, 1925, Table III, Column 3, for *In 2.5 per cent NaOH* read *In 2.5 per cent HCl*.

For $[\alpha]_D^{25}$ of epichondrosaminic acid in 5 per cent NaOH for -1.8 ; $c = 2.5$ read $+1.8$; $c = 2.5$.

acids in the *d* or *l* series is based on the direction of the change in rotation from the free acid to its salt.

It was pointed out in a previous article that the sugar acids of which the carbon atom 2 has the configuration of that of gluconic acid rotate to the right when in the form of metallic salts and to the left, when in the free state.² In mannonic acid and the acids having carbon atom 2 of the same configuration, the rotations are reversed. In four of the 2-aminohexonic acids of the *d* series³ (the two arabino- and the two xylohexosaminic acids) the directions of rotation of the free acids and of their sodium salts are similar to those of the corresponding hexonic acids. In the

TABLE III.

Acids.	In 5 per cent NaOH. $[\alpha]_D^{20}$	In 2.5 per cent NaOH. $[\alpha]_D^{20}$
Chitosaminic.....	+1.3 " = 5.0.	-15.0
Epichitosaminic.....	-5.0 " = 5.0.	+10.0
Dextro- <i>d</i> -xylo-2-aminohexonic.....	-16 " = 2.5.	+14.0
Levo- <i>d</i> -xylo-2-aminohexonic.....	+2.0 " = 2.5.	-11.0
Chondrosaminic.....	-15 " = 2.5.	-17.0
Epichondrosaminic.....	-1.8 " = 2.5.	+8.0
Dextro- <i>d</i> -ribo-2-aminohexonic.....	+2.0 " = 2.5.	+12.5
Levo- <i>d</i> -ribo-2-aminohexonic.....	-15.0 " = 2.5.	-26.0

remaining four (the two lyxo- and the two ribohexosaminic acids) the direction of rotation is the same for the salt and for the free acid. Thus these four acids behave seemingly differently from the corresponding hexonic acids. But if, instead of the direction of rotation, the magnitudes of rotation are compared, it is seen that in all the 2-aminohexonic acids the differences in the rotations of the salts and corresponding acids are similar to those in the hexonic acids; namely, in four the salts have a higher dextro-rotation than the free acids and in the other four the conditions are reversed. On the basis of these considerations, the hexosaminic and the hexonic acids can be classified in the following two groups.

First Group.

Chitosaminic acid.

Chondrosaminic acid.

d-Levoxylohexosaminic acid.

d-Levoribohexosaminic acid.

Gluconic acid.

Galactonic acid.

Gulonic acid.

Allonic acid.

² See Table II.

³ See Table III.

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Second Group.

Epichitosaminic acid.

Epichondrosaminic acid.

d-Dextroxylohexosaminic acid.

d-Dextroribohehexosaminic acid.

Mannonic acid.

Talonic acid.

Idonic acid.

Altronic acid.

Thus it is seen that on the basis of this classification (the direction of change of rotation between salt and acid), the conclusions arrived at on the basis of the consideration of the 2-aminohexonic acids coincide with those arrived at on the consideration of chitosamine and epichitosamine.

If the classification given in Table III is accepted, it follows that in the α -aminohexonic acids the deamination by means of nitrous acid takes place without Walden inversion.

The latter conclusion is in harmony with the view expressed recently by Freudenberg in regard to α -amino acids. Freudenberg accepts a relationship of the α -amino to α -hydroxy acids similar to the one expressed here regarding hexonic and 2-aminohexonic acids. (See also Clough.)

Finally, the conclusion finds direct corroboration in the observations of Levene and Mikeska on α -thio and α -sulfonic acids. In the thio and in the sulfonic acids of the same configuration the exchange of the hydrogen in the carboxyls by metal ions is accompanied by a change of rotation in the same direction. Thus the dextrothiopropionic and the dextrosulfopropionic acids show a change of rotation to the left when the carboxylic salt is formed. In the levo acids the conditions are reversed.

Thus it seems that the classification given above has more arguments in its favor than that of Table I and, therefore, the view that chitosamine has the configuration of glucosamine, and chondrosamine that of galactosamine may be more justified today than the converse view.

CONCLUSION.

1. There seems to be sufficient reason to classify such sugar acids with the group of *d*-gluconic acids (with respect to configuration of carbon atom 2), the salts of which show a higher dextrorotation than the free acids.

2. It seems justified to assume that in the 2-aminohexonic

acids the same rule holds as in (1), and that the classification given above has the preponderance of evidence in its favor.

3. It seems probable that in the group of 2-aminohexonic acids the deamination by means of nitrous acid takes place without Walden inversion.

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REPRODUCTIVE POTENCY OF DRY MILK AS AFFECTED BY OXIDATION.

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(Received for publication, December 26, 1924.)

The frequently unsuccessful reproduction and rearing of white rats on synthetic rations have resulted in numerous investigations designed to determine the particular inadequacies of such rations. Although there is quite general agreement as to the necessity of optimal amounts of vitamins A and B (1, 2, 3), there is, nevertheless, certain evidence which indicates that seemingly adequate amounts of these factors do not always allow successful reproduction and rearing of the young. These apparent discrepancies have led to the consideration of suitable balances of the constituents of the ration as a possible factor to be reckoned with. Anderegg (4) states that if the proportion of fat to protein is too high, growth may be normal in the first generation, but the animals fail to reproduce. Hartwell (5) indicates that excessive protein in the diet of the mother during lactation is detrimental to the suckling. Mattill and coworkers (6) find that on a milk diet low in fat and without added lard, reproductive failure does not occur. These investigators have given particular attention to the matter of reproduction when milk, either fluid or dried, was the variable component of the ration. They claim that the inability of the rats to reproduce on a milk diet high in fat is not removed by the addition of 2 to 5 per cent of nuclein-containing proteins or 2 per cent of vitamin B (Harris), but when supplemented with 5 to 10 per cent of wheat embryo failure does not occur.

Evans and Bishop (7), after careful investigations in which the usual observations are supplemented by numerous anatomical examinations, are led to the belief that there is a distinct factor, which they designate as X, which is necessary for reproduction.

They report that this factor is present in lettuce, egg yolk, meat, and the alcoholic extract of wheat embryo, but practically absent in milk. They claim there is a definite but low amount of the substance in milk fat. While the evidence recorded by these investigators may seem to indicate the existence of an unknown factor X such a hypothesis is not yet generally accepted.

In view of the lack of common agreement as to those factors which a synthetic ration must furnish to insure regular and normal reproduction and rearing of the young, it is considered opportune to record the results of certain preliminary investigations which it seemed advisable to conduct before proceeding with more extended experiments on the matter of fertility.

EXPERIMENTAL.

During the course of nutritional studies on dry milk we have had occasion to give particular attention to the deteriorating effects of oxidation during prolonged storage. The data recorded hereinafter were obtained with dry milk which had been stored for approximately 2 years under conditions which permitted variable degrees of oxidation. The dry milk used was a part skimmed product manufactured by the Just process and contained 12 per cent fat.¹ It was prepared from milk produced during the early fall while the cows were still on pasture although stall feeding supplemented the pasture grass to a certain extent. Immediately after desiccation the milk was divided into 3 parts and the different portions stored under the following conditions. One portion was sealed in gas-tight tin containers in which the enclosed gas was air. Another portion was sealed in an atmosphere of carbon dioxide by a procedure which at that time was supposed to represent satisfactory achievement in the commercial use of the method. The third portion was packed by a method wherein a dry oxygen-absorbing material was introduced into the filled container before it was sealed. Previous tests had shown that this method was efficacious in removing all but a small fraction of 1 per cent of the oxygen of the enclosed air within a few hours. The powder packed in this manner may therefore be considered as having been held in an atmosphere of deoxygenated air. All samples were held at normal room temperatures which fluctuated from 60° to 85°F.

In order to have a suitable check on the gaseous environment of the powder frequent analyses were made on the gas content of representative cans during the first 6 months of the storage period. At the time the powder was packed numerous determinations showed an average oxygen content of the normal air to be 20.7 per cent. After 6 months storage the oxygen content of the gas enveloping the air-packed powder was found to vary

¹ This was the Dryco Brand.

from 15.5 to 19.6 per cent. This clearly indicated the absorption of oxygen by the milk powder and further examination revealed the results of characteristic oxidative changes. Analysis of the gas in representative cans packed by the carbon dioxide method showed at the beginning of the storage period an average oxygen content of 0.47 per cent with variations between 0.25 and 0.85 per cent. After 6 months' storage the oxygen content of the enveloping gas showed an average of 0.25 per cent with variations between 0.1 and 0.4 per cent. Analysis of the gas not carbon dioxide showed an average oxygen content of 13.6 per cent with variations between 9.0 and 18.1 per cent. If it may be assumed that the gas not carbon dioxide in the cans at the beginning of the storage period was air with a normal oxygen content, these results indicate oxygen absorption by the powder from originally impure carbon dioxide. Similar analyses were made on representative cans packed with the oxygen-absorbing material. The average initial oxygen content of the gas in these cans was 0.22 per cent with variations between 0.03 and 0.34 per cent. After 6 months' storage the gas in the representative cans packed by this method showed an average oxygen content of 0.24 per cent with variations between 0.1 and 0.4 per cent. These results indicate that no oxygen was taken up by the powder packed with the oxygen-absorbing material. The slight traces found at the beginning and after storage may be attributed to analytical error. Examinations of the powder after 2 years seemed to warrant this assumption as there was no evidence of characteristic oxidative changes. Examination of the samples packed by the carbon dioxide method, however, revealed slight evidence of such changes, although much of the product showed no evidence of oxidation.

The above records are included as relevant data for the proper interpretation of the results obtained from the following tests, particularly those showing differences in reproductive ability as affected by the degree of oxidation to which each of the powders has been subjected.

White rats, approximately 25 days old, which had been reared in our Laboratory from females on an adequate stock ration (8), were used. The animals for the various tests were litter mates, and unless otherwise noted, two males and two females were constantly together in standard cages in a well lighted room with southern exposure. Observations on growth and reproduction were recorded periodically. All data are reported without selection.

The experimental rations all consisted of casein, extracted with alcohol for 9 days and subsequently heated for 1 week, 18; salt No. 40 (9), 4; agar-agar, 2; dried brewer's yeast, 3; the milk powder in varying amounts as indicated below; and dextrin to make the 100 parts. Each of the milk powders after about 20 months' storage was incorporated in the ration on a basis approximating the equivalent of 10, 15, 20, and 30 cc. of reconstituted milk per rat per day. Assuming an average consumption of 60 gm. per week per rat these amounts correspond to 11.7, 17.5, 23.3, and 35 per cent of the ration. As this milk powder contained only 12 per cent fat the above amounts correspond to 1.4, 2.1, 2.8, and 4.2 per cent of butter fat, respectively. The graphic results of these experiments are given in Charts 1 to 4.

The outstanding feature in Charts 1, 2, 3, and 4 is the striking difference in reproduction obtained on rations containing milk powder stored in air (Lots 1, 4, 7, and 10), or that packed with the carbon dioxide method (Lots 2, 5, 8, and 11), and on those containing the milk powder stored in the deoxygenated air (Lots 3, 6, 9, and 12). It will be noticed that there was no reproduction on any of the air-packed or carbon dioxide-packed powder although resorptions are in some cases indicated. Nevertheless, on even as low as 11.7 per cent of powder stored in the deoxygenated air, reproduction was possible. Only one young was seen in this case, however, and it was almost immediately destroyed by the mother. Higher amounts of this same dry milk produced relatively better results as are shown in Charts 2, 3, and 4 (Lots 6, 9, and 12). A deficiency is indicated when 17.5 per cent was used in that the litters were small, two to four, and lived only a few days. On 23.3 per cent Chart 3, Lot 9, the litters were practically normal and although the first litters were not reared, one of the second litters was successfully raised through the weaning period. This generation, still on the same ration after 12 weeks, shows an average weight of 174 gm. for the males and 130 gm. for the females. Chart 4, Lot 12, gives the results of feeding 35 per cent or 4.2 per cent of butter fat equivalent of the same milk powder. In this case the litters were all normal and all were reared through the weaning period. None of these animals were kept after the weaning period. The significance of the foregoing results lies in the fact that all the constituents of the rations for the different groups were identical with the exception that the milk powder was packed and held under different conditions which allowed variable degrees of oxidation.

As a further check on these results other tests were carried out in which relatively fresh dry milk, butter fat, and cod liver oil were the variable components of the ration. This dry milk was of the same composition as that in the previous tests and was produced at the same factory during the same season of the year. The same amounts as for the stored powder tests were used. Although the powder was about 7 months old at the time the experiments were concluded, the results were substantially the same as those obtained from the powder stored in deoxygenated air. There was no reproduction, however, on the 11.7 per cent

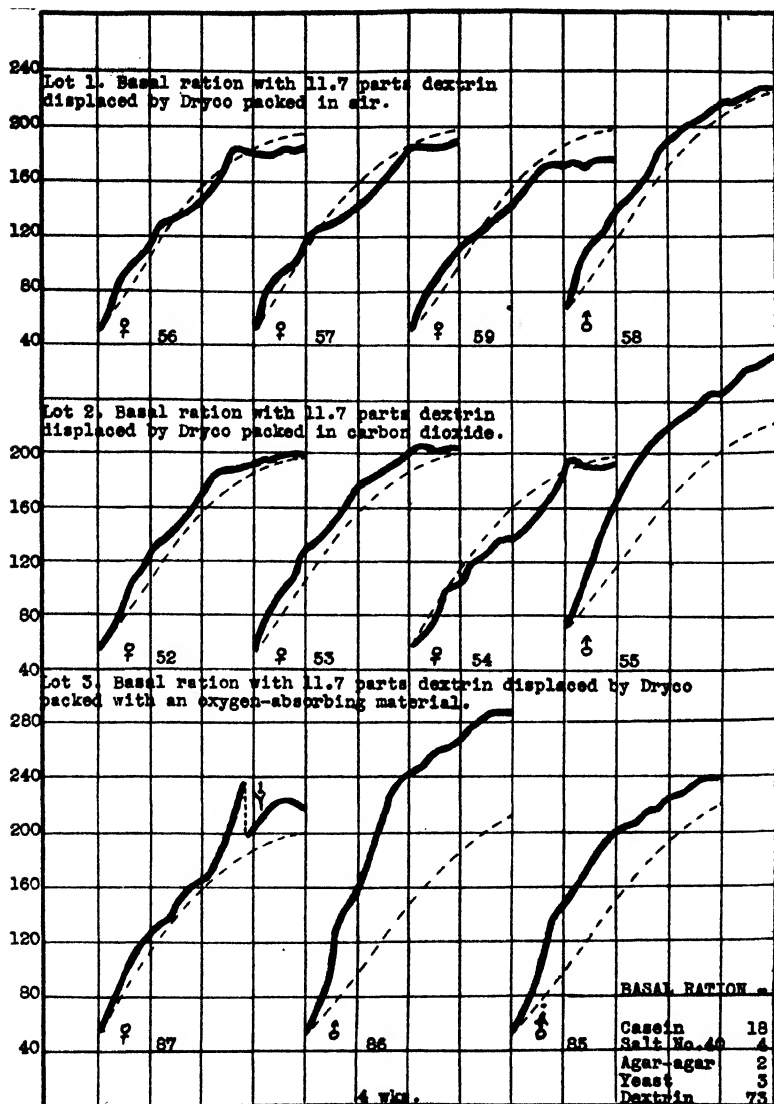


CHART 1. This chart shows that milk powder as 11.7 per cent of the ration is inadequate for successful reproduction. Only one young was born in Lot 3 and it was destroyed immediately by the mother. No young were born in Lots 1 and 2.

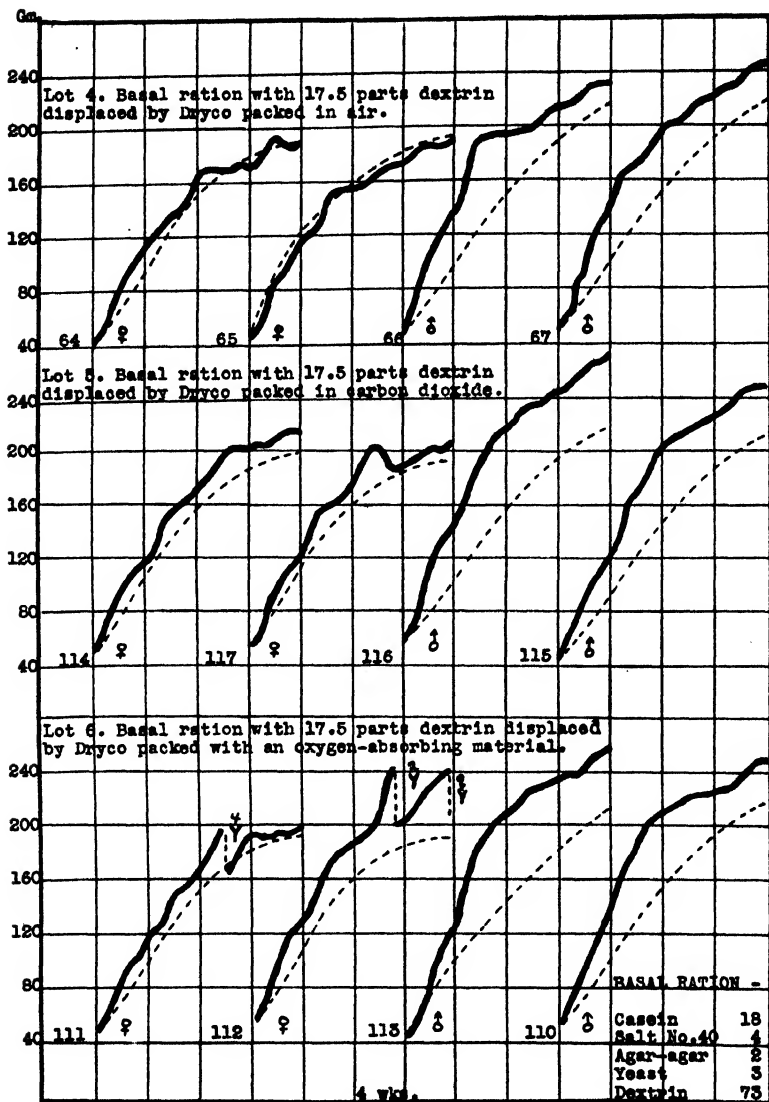


CHART 2. This chart shows that with the milk powder as 17.5 per cent of the ration only those fed the preserved product bore young. Again there is no reproduction when the milk powder had been stored in air or carbon dioxide. Curves for Rats 64 and 117 indicated resorptions.

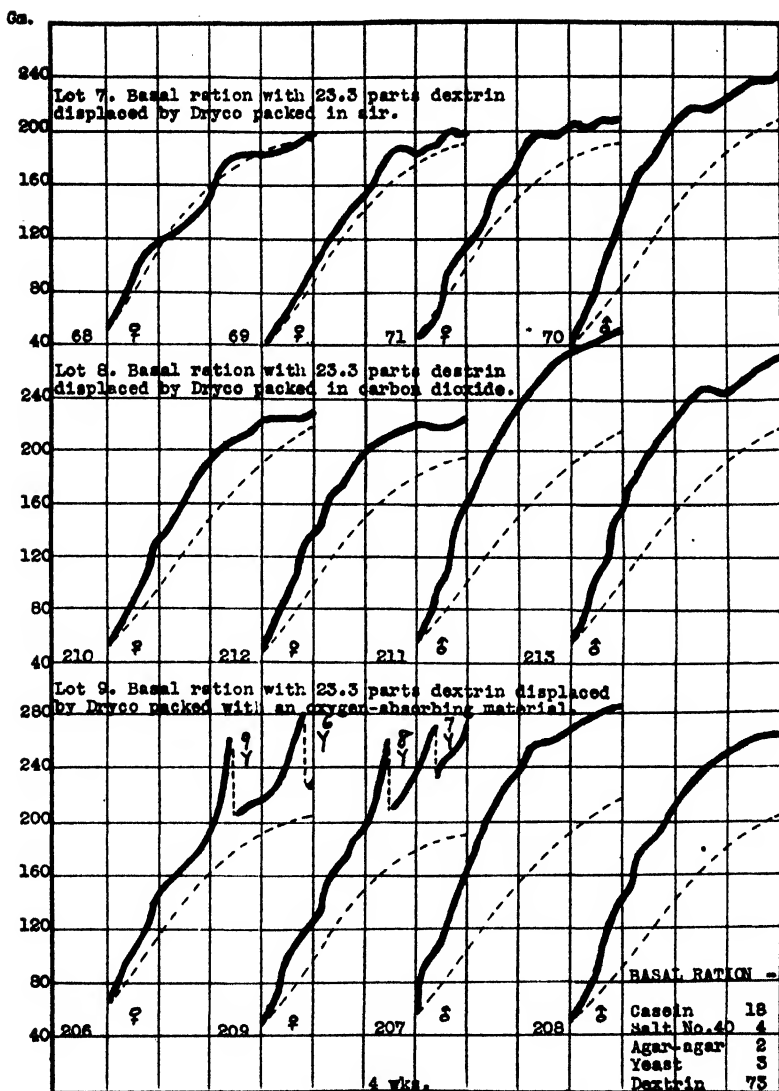


CHART 3. This chart shows a marked contrast between powder packed in the absence of oxygen and that packed in air or carbon dioxide. One litter fed on the ration containing the preserved powder is still alive and normal at the end of 12 weeks. There was no reproduction on either of the other rations.

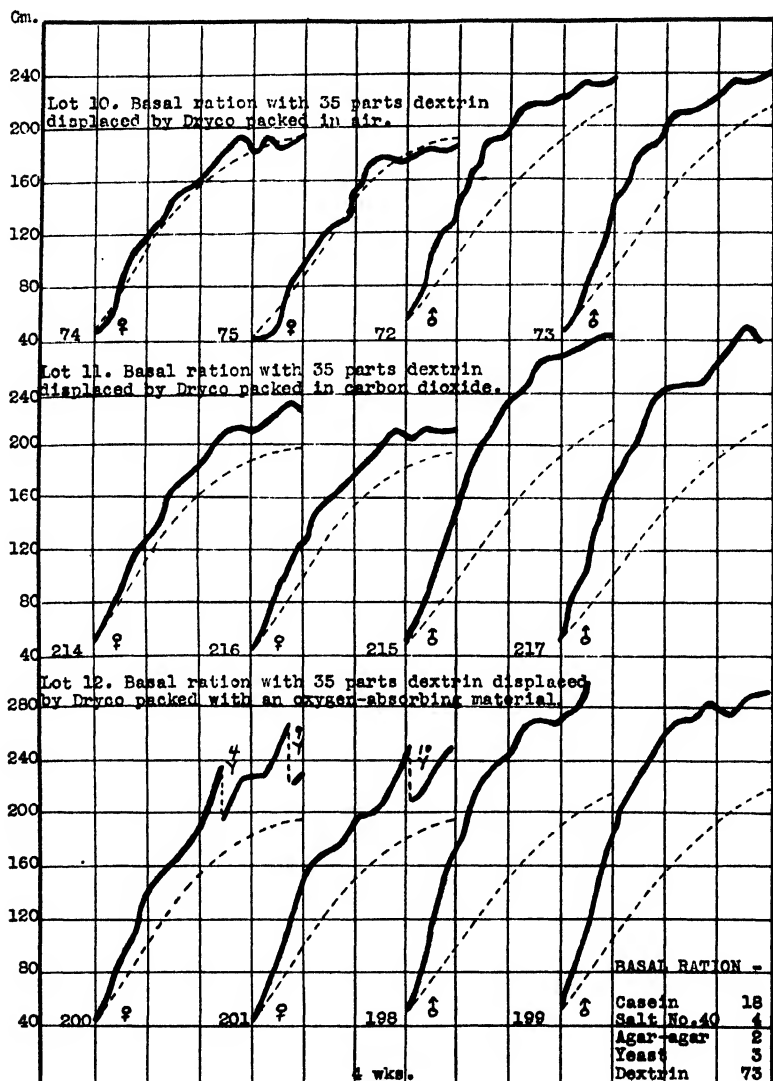


CHART 4. This chart shows the remarkable effect of oxidation of milk powder upon reproduction. The quantity used appeared adequate for normal reproduction and rearing of young if properly preserved, as is shown by the results from that packed in deoxygenated air. Similar quantities stored in air or carbon dioxide gave no better results than the lower quantities held under the same conditions.

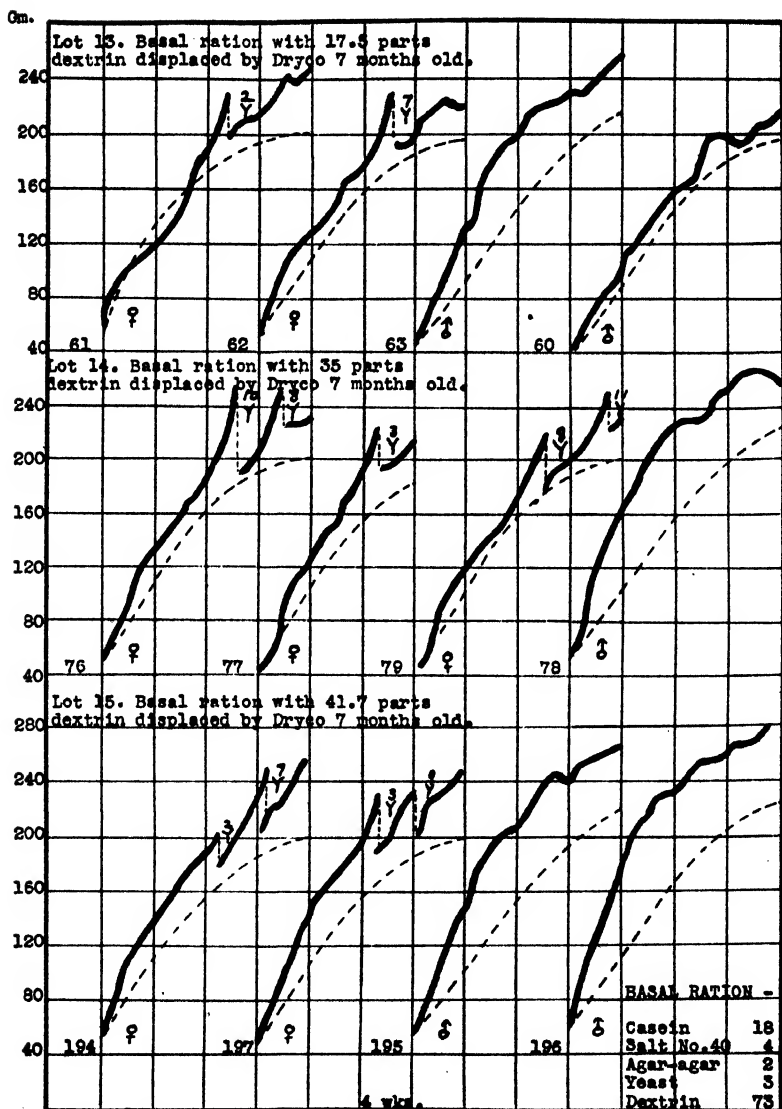


CHART 5. This chart indicates what may be expected when 7 months' old powder is used in varying quantities in the ration. Normal reproduction and rearing of the young was possible when this powder constituted 41.7 per cent or 5 per cent of butter fat equivalent.

quantity. It required 5 per cent of butter fat equivalent or 41.7 per cent of the milk powder to produce and rear young equal in size and vigor to those produced by 4.2 per cent of butter fat equivalent or 35 per cent quantity of the milk powder packed in the deoxygenated air immediately after manufacture and held in this manner for about 2 years. Inasmuch as this powder was about 7 months old when the experiments were completed and was stored in an atmosphere of air during this period, it is quite possible that slight oxidation may have taken place. The results are shown in Chart 5.

When butter fat was used as 5 per cent of the ration to replace the butter fat of the dry milk and with no other milk solids except the purified casein, young were produced and reared through the weaning period. The young were not vigorous, however. It is significant in this connection to note that in preparing this butter fat no especial measures were taken to prevent oxidation. Butter obtained from the market was melted, centrifuged, and filtered through filter paper in a hot water-jacketed funnel, cooled immediately, and stored in glass-stoppered bottles. Although the time required for its preparation was comparatively short, the exposure to heat and air undoubtedly offered opportunity for limited oxidative changes.

The results obtained when 2 per cent of cod liver oil was substituted for butter fat were significant in that there was no reproduction. Two lots of cod liver oil were used. One was over 3 years old and of evidently inferior quality as shown by its dark color and strong odor. The other was a fresh lot of certified cod liver oil and of apparently excellent quality. Both lots were no doubt somewhat objectionable to the rats as the food consumption was lower on these rations than when butter fat or milk powder was used. Even though 2 per cent of cod liver oil presumably furnishes an adequacy of vitamin A there was no reproduction in either of the lots. Reproductive failure on cod liver oil is also reported by Evans and Bishop, even in quantities as high as 25 per cent of the ration. The rats on the old oil developed a slight eye infection after 14 weeks which, though not serious, continued throughout the experiment.

The data recorded in this preliminary report do not permit definite statements regarding all prerequisites necessary for suc-

cessful reproduction. The interpretation of the results cannot be construed as tending to disprove the existence of a distinct reproductive factor X, nor, in the light of present knowledge, do they necessarily tend to corroborate the existence of such a factor. If further investigations should prove beyond doubt the existence of a distinct vitamin necessary for reproduction it is apparent from our data that this factor is readily susceptible to oxidation in the condition in which it exists in dry milk. On the other hand, if the destructive oxidative changes should prove to have been limited merely to the quantitative reduction in potency of vitamin A it would appear that the marginal requirements of this vitamin necessary for successful reproduction are greater than heretofore appreciated. While variable amounts of milk powder were used in these tests it is believed that the interpretation of the results obtained with the various quantities must limit the primary function of the milk powder to that of a vehicle for the necessary prerequisite, rather than to proper balances of fat, protein, and salts.

SUMMARY.

The results of a preliminary investigation are reported wherein it is shown that oxidative changes which may take place under conditions prevailing in milk powder, stored in air over long periods, prevent reproduction.

Milk powder containing 12 per cent of fat, packed and held in an atmosphere of air in hermetically sealed containers for nearly 2 years, does not permit reproduction in white rats when fed in quantities varying from 11.7 to 35 per cent of the ration.

The same powder stored in deoxygenated air for the same period allowed normal reproduction and the rearing of young when fed in quantities varying from 23.3 to 35 per cent of the ration. Reproduction was possible on lower quantities, but rearing of the young was not successful.

The same powder stored in an atmosphere of carbon dioxide as packed by a commercial method gave no better results in the matter of reproduction than the powder stored in an atmosphere of air. The oxygen content of the gas enveloping this powder was found to be less than 1 per cent at the time of packing, and subsequent analyses show the absorption of some of this oxygen.

Therefore, it cannot be stated whether the negative results obtained with this powder were due to the carbon dioxide or to the oxygen impurity.

Milk powder of the same brand made under the same conditions at the same time of year as that for the storage experiments but held in an atmosphere of air for 7 months allowed reproduction and successful rearing of the young when fed as 41.7 per cent of the ration. Reproduction on smaller quantities was possible, but rearing of the young was not always successful.

Successful reproduction and rearing of the young resulted when the butter fat, as supplied by the Just process dry milk, comprised from 2.8 to 5 per cent of the ration, providing the milk powder had not been subjected to excessive oxidation after manufacture.

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PRESENCE OF THE ANTISCORBUTIC SUBSTANCE IN THE LIVERS OF CHICKENS FED ON SCORBUTIC DIETS.*

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Numerous investigations have been made showing the importance of fat-soluble vitamins and water-soluble vitamin B in the nutrition of fowls. Only a few reports, however, have been published concerning the place of water-soluble C in the nutrition of avian species.

Hart, Halpin, and Steenbock (1), working with growing chicks, state: "It appears that this species requires a liberal supply of the vitamins of cod liver oil during its most active period of growth, but that the water-soluble and antiscorbutic vitamin requirement can be met by the amounts contained in a cereal grain and skimmed milk."

Emmett and Peacock (2) suggest in a preliminary report that the vitamin C requirements of the chick appear to be much less than those for vitamins A and B. These investigators (3) later fed synthetic diets, free from vitamin C, to chicks which grew at a normal rate. Eggs were laid at 187 days and the fowls were kept on this diet until they weighed 1,387 gm. To other chicks they fed the same diet supplemented with a potent tomato extract with more than a sufficient amount of vitamin C to prevent scurvy in guinea pigs. This additional vitamin had no effect in stimulating the growth or activity of the chicks during 40 days. They conclude: "...during the early stages of the growth of the chick, the vitamin C, contrary to the notion of most feeders, was not essential. Whether its presence is needed later is not evident from these data."

Mitchell, Kendall, and Card (4) fed to chicks diets of corn products and tankage, generally recognized as having no vitamin C. After 12 weeks, part of the chicks received a vitamin C supplement in the form of tomato juice, and others received tomato juice in which the vitamin C had been destroyed. From these tests they concluded that the chicken is not susceptible to scurvy and, therefore, may thrive on diets devoid of vitamin C.

* Published with the approval of the Director of the Agricultural Experiment Station, Purdue University, Lafayette.

Plimmer, Rosedale, and Raymond (5) kept chickens for nearly 14 months on a diet of white rice and dried skim milk, supplemented with cod liver oil and marmite. They report: "Though success in raising a new generation has been very limited, which appears to be connected with some other deficiency in the diet, it may still be concluded that chickens, pigeons and other birds never seem to require the addition of C-vitamin to their food."

Sugiura and Benedict (6) have successfully reared pigeons which produced young on synthetic diets containing no antiscorbutic vitamins.

Shorten and Ray (7), in working with fowls averaging 1,600 gm., fed a basal ration of rice, supplemented with various sun-dried vegetables, and found no apparent effect upon the fowls, although certain of the supplements used furnished little or no vitamin C as shown by experiments with guinea pigs.

One of us (C. W. C.) has been able to keep mature cockerels of American varieties confined in coops $2 \times 2 \times 2$ feet for periods of 280 days without the addition of vitamin C to the diet. At the end of that time two birds were autopsied, but no indications of the lesions of scurvy were apparent. So far as we have been able to find, no one has reported lesions of scurvy occurring in chickens.

From the review of the literature it is evident that fowls of various ages have little apparent need for vitamin C in their diet. It was the purpose of this investigation to determine whether vitamin C occurred in the tissues of the fowl's body.

EXPERIMENTAL.

In a similar manner as Parsons (8), Parsons and Hutton (9), Lepkovsky and Nelson (10), and others have studied the antiscorbutic requirements of the rat and guinea pig by demonstrating the absence or presence of the antiscorbutic substance in their tissues, so also was this experiment planned to show the absence or presence of the antiscorbutic vitamin in the livers and kidneys of chickens which had been fed on a scorbutic diet for over 3 months.

A group of cockerels of the American varieties was used, which had been fed on a ration consisting of degerminated yellow corn, tankage, salt mixture, and yeast. All of these birds had suffered from polyneuritis in another experiment, but were completely cured before being used in these tests.

The determination of the persistence of the antiscorbutic vitamin in the livers and kidneys of these birds was made by testing its curative action when fed to guinea pigs suffering from well defined scurvy.

The scorbutic ration used in feeding the guinea pigs was similar to that

employed by Parsons (8) except that agar-agar was used instead of filter paper. The ration had the following composition.

	gm.
Autoclaved soy bean meal.....	84
NaCl.....	3
Ca lactate.....	3
Yeast.....	3
Butter fat.....	5
Agar-agar.....	2

A selected group of guinea pigs was fed on this ration until the development of marked symptoms of scurvy. With the onset of scurvy, the guinea pigs ceased eating. This condition was followed by the customary loss in weight, and accompanied by the other characteristic symptoms. Daily doses of livers or kidneys were then administered.

TABLE I.
Table Showing Autopsy Findings in the Guinea Pigs.

Guinea pig No.	Bone lesions.				Hemorrhage.			
	Jaw.	Teeth.	Ribs.	Joints.	Muscles.	Intestines.	Joints.	Ribs.
1	+++	++++	++++	++++	++++	++	++++	++++
2	++	++++	++++	++++	++++	++	++++	+++
3	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—
5	+	+	+	+	—	—	—	—
6	++	—	—	+	—	—	—	—
7	+	—	—	+	—	—	—	—
8	—	—	—	—	—	—	—	—

In the preparation of the livers and kidneys, great care was exercised always to have a fresh supply and to feed accurate amounts to the animals. The birds were killed as required to furnish the necessary daily doses of livers or kidneys. These organs were made into suspensions by macerating weighed portions in a mortar and finally adding water to the paste until such a consistency was reached as could be fed with a pipette. The guinea pigs consumed this greedily.

RESULTS.

The results of this experiment are recorded in Table I and Chart 1.

Guinea Pigs 1 and 2 received no additions to their scorbutic ration. They succumbed to very severe scurvy. Nos. 3 and 4 received 15 gm. of liver daily. On the chart this was begun at a

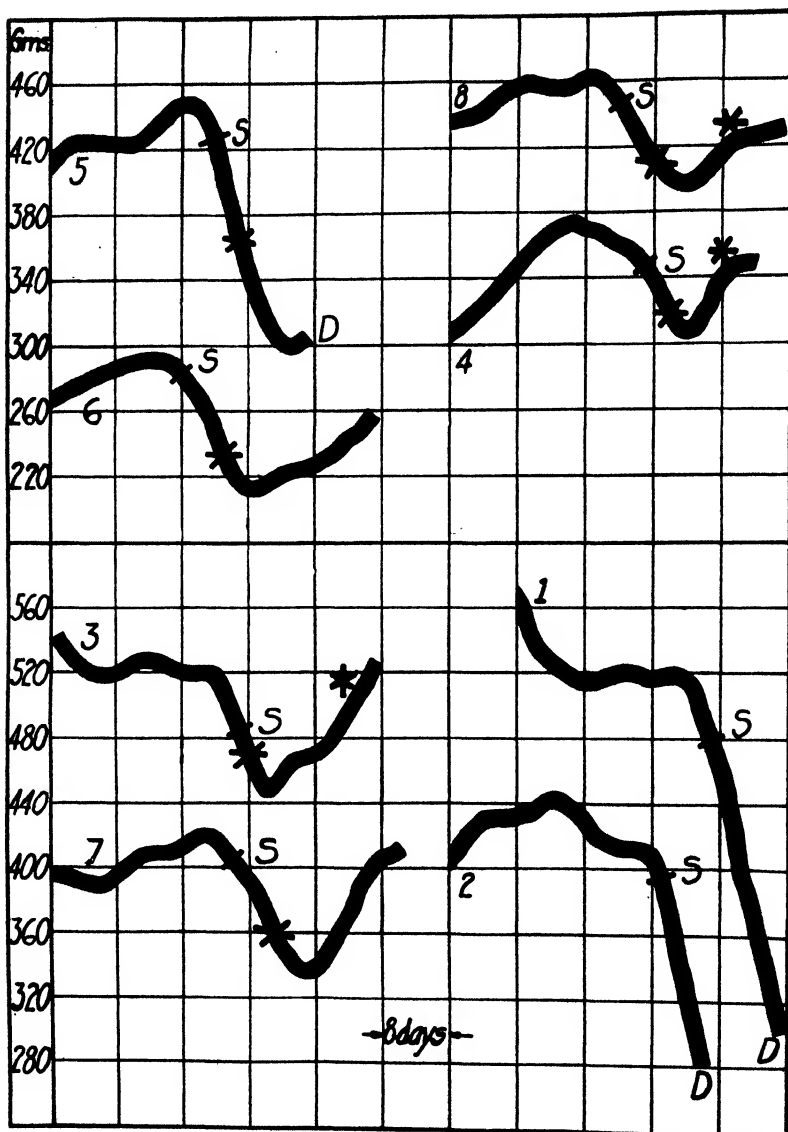


CHART 1. This chart shows the effect of water suspensions of livers and kidneys from fowls on a scorbutic diet when fed to guinea pigs suffering from scurvy. Scurvy is indicated by *S*; feeding of suspensions began at the point indicated by a cross (*X*); reduction of amount was made at the point indicated by an asterisk (*).

point marked by a cross (X). Later this amount was decreased to 5 gm. daily as indicated by an asterisk (*). No. 5 received 10 gm. of liver daily. On the 8th day it choked on the suspension and died. The cause of death was confirmed by autopsy examination. For some distance from the hilus of each lung, all the lobes were solidified in a state of red hepatization. All hemorrhages had disappeared, but the bones still showed slight fragility. No. 6 also received 10 gm. of liver daily. An advanced stage of scurvy was allowed to develop in this animal before the suspension was fed. As a result, the bones in one of its fore legs became so fragile that it broke under the animal's weight. Complete recovery was secured. No. 7 was given 5 gm. of liver. No. 8 received 10 gm. of kidney which was later reduced to 5 gm. daily. A dosage of 5 gm. of liver daily appeared to be as effective as greater amounts.

DISCUSSION.

It has been common observation that the rat would thrive on diets which contained no demonstrable amounts of antiscorbutic substance as determined by feeding to guinea pigs, a species very susceptible to scurvy. Parsons (8), however, was the first to show that the liver of the rat contained the antiscorbutic substance. She demonstrated the presence of this substance not only in the liver of rats fed for a short time on a diet rich in antiscorbutic substance, but also in those fed over a long period of time on a scorbutic diet. Similar results were obtained by Lepkovsky and Nelson (10) who found that in a second generation of rats on a scorbutic diet the livers were as rich in the antiscorbutic substance as those of the first generation. Parsons' results were interpreted as "an indication of the need for the antiscorbutic factor in the normal metabolism of the rat. . . . It seems entirely improbable that the presence of this factor in the bodies of these animals should be accidental, especially since the amounts in the bodies of the two sets of rats approximate each other so closely."

Parsons (8) suggested several hypotheses to account for these results. One of these was that the quantity of antiscorbutic substance required by the rat was very small, but might be met by diets very low in this factor on which the guinea pig acquires scurvy. This hypothesis was rendered less probable by a later

investigation by Parsons and Hutton (9) where two successive generations of rats were produced and reared on purified rations without noticeable difference in the antiscorbutic content of their livers.

Parsons (8) further pointed out: "It is conceivable that the liver cells are so constituted that a considerable supply of the antiscorbutic factor may be contained in them while at the same time the content of other tissues is below the level at which proper functioning is possible." This hypothesis was considered very improbable by a later investigation (11) when it was demonstrated that the livers of scorbutic guinea pigs were depleted of the antiscorbutic substance, while those from normal guinea pigs contained an abundance of this factor.

Another hypothesis proposed by Parsons in her initial investigation (8) was that the rat may synthesize the antiscorbutic substance, although this may be a conversion of some inactive form of the vitamin by such physiological processes as the sprouting of seeds. Chick and Delf (12) have shown that the antiscorbutic substance can be developed to a substantial quantity in seeds by germination. Parsons and Reynolds (11) are inclined toward this last mentioned hypothesis after further investigation.

It would appear from our findings and from those of others that, as far as the antiscorbutic requirement is concerned, the chicken is analogous to the rat. Our data, however, do not throw any light on the method of acquirement of the antiscorbutic substance in the liver or kidney of the chicken.

It is possible that the mature chicken may carry a store of vitamin C for a very long period and that only a small amount is needed by the chicken. Such a storage or economy apparently occurs in the case of the fat-soluble A which has been shown many times to be essential for growth of young chicks (1, 3, 5). But one of us (C. W. C.) has been able to keep a mature male bird in confinement for 218 days on a diet of whole white corn or degerminated white corn and yeast, which could contain only a trace of fat-soluble A. While this bird had lost some weight he appeared normal in other respects.

It has been pointed out by Steenbock, Sell, and Nelson (13) that the liver of the rat acts as a storage for the fat-soluble vitamin and that the amount of this substance in the liver varies with the

diet. Osborne and Mendel (14) observed a similar influence of diet on the water-soluble B content in the liver of the rat. They fed dried livers of rats declining on a diet free from water-soluble B. Rats receiving as high a dosage as 200 mg. daily either failed to make adequate gains or declined and died. When dried livers from rats on a wholesome mixed diet were fed at 50 mg. a response was shown; 100 mg. gave almost a normal rate of growth. Findlay (15), however, fed hens on a diet of polished rice, on alternate days giving each 1 gm. of the organs, including liver, of a pigeon dying of polyneuritis. He found no histological or other evidence of polyneuritis in the hens at the end of 50 days. The usual time for development of polyneuritis, under such conditions, is from 25 to 30 days. Findlay does not state whether his material was dried, but if it were (which is improbable), the dosage, considering body weight, was less for his hens than the dosage given by Osborne and Mendel to their rats when feeding 200 mg. of the liver from declining rats. Furthermore, we have found in other studies¹ that mature cockerels require for protection against polyneuritis over three times the level of a certain yeast which will permit normal growth in rats. Evidently the livers of Findlay's polyneuritic pigeons were quite rich in the antineuritic substance and it is not inconceivable that, unlike the rat, the avian species may retain in their organs considerable of this factor, though the diet be so deficient as to cause decline, polyneuritis, and death.

It is possible that our male birds, after being on a scorbutic diet for over 3 months, may have carried over sufficient water-soluble C in their livers to bring about the results we have obtained.

Unfortunately there are no available data showing the variation in the water-soluble C content of livers from chickens on scorbutic and antiscorbutic diets. We are not aware that a second generation of chickens has been reared with any degree of success on a purified diet free from the antiscorbutic substance. We are planning some further investigations along these lines. Until such information is gained, definite conclusions cannot be drawn concerning the relation of water-soluble C to this species.

However, from the work of others and from our own findings,

¹ Unpublished data.

we are inclined to accept for the chicken the hypothesis that Parsons has formulated for the rat; namely, that this species produces the antiscorbutic substance in metabolism from some source not available to the guinea pig.

We wish to acknowledge our indebtedness to Mr. L. P. Doyle of the Veterinary Department of this institution for his assistance in making the postmortem examinations on the guinea pigs.

SUMMARY.

1. Fowls show no apparent ill effect after several months of deprivation of antiscorbutic substance.

2. Cockerels which have been fed on a scorbutic diet for an extended period still have the antiscorbutic substance in their livers and kidneys.

3. The abundant supply of the antiscorbutic substance suggests that it is necessary for normal metabolism.

4. Possible explanations for the presence of the antiscorbutic factor in the tissues of fowls have been presented.

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STROPHANTHIN.

VI. THE ANHYDROSTROPHANTHIDINS AND THEIR BEHAVIOR ON HYDROGENATION.

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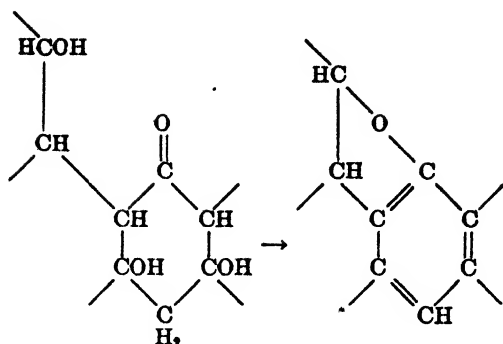
In a previous communication¹ it has been shown that when strophanthidin is subjected to the action of absolute alcoholic hydrochloric acid, dehydration occurs with simultaneous formation of an ethylal derivative, presumably with γ -oxidic structure. Depending upon the conditions employed, either 1 or 2 mols of water are eliminated with the production of derivatives of mono- or dianhydrostrophanthidin, respectively. Subsequent hydrolysis results in the formation of the hydroxyketones. Although these substances apparently exhibit in their behavior toward permanganate in acetone solution no greater degree of unsaturation than strophanthidin itself the formation of olefinic linkings has been definitely established by the present hydrogenation experiments. It was hoped, incidentally, after hydrogenation of the unsaturated groups, to reduce further the ketonic group and the remaining hydroxyl group and ultimately the lactone group with formation of the fundamental saturated hydrocarbon upon which strophanthidin is built. But the unexpected course which the hydrogenation experiments with dianhydrostrophanthidin have taken has rendered difficult the complete realization of this plan.

Owing to certain observations which will be discussed below, attempts were made to dehydrate further dianhydrostrophanthidin. If this substance is dissolved in concentrated hydrochloric acid 1 mol of water is removed with the formation of trianhydrostrophanthidin, $C_{23}H_{26}O_3$. This substance, while still possessing the lactone group, no longer reacts with ketone reagents

¹ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lix, 713.

and no longer yields an acyl compound. It would seem, therefore, that its formation is due to the further removal of water from the oxidic form of dianhydrostrophanthidin. An oxidic structure, and possibly one of γ -oxidic nature, must be accepted. Although it seems probable that a new olefinic linking is formed it was possible only indirectly to establish this by hydrogenation experiments.

The behavior of this substance towards palladium and hydrogen has been quite remarkable. Contrary to the ease with which dianhydrostrophanthidin is hydrogenated, trianhydrostrophanthidin very slowly absorbs but 1 mol of hydrogen in a manner suggestive of the behavior of strophanthidin itself. Apparently a single substance only was formed, dihydrotrianhydrostrophanthidin, $C_{23}H_{28}O_3$. It was definitely established that this substance is produced by saturation of the double bond originally present in strophanthidin in the following manner. Dihydrostrophanthidin was converted into dihydrodianhydrostrophanthidin over the ethylal. This was in turn subjected to the action of concentrated hydrochloric acid. The resulting substance, $C_{23}H_{28}O_3$, proved to be identical with the dihydrotrianhydrostrophanthidin obtained by hydrogenation of trianhydrostrophanthidin. The failure of the three olefinic linkings of trianhydrostrophanthidin to respond to hydrogenation, as in the case of dianhydrostrophanthidin, seems explainable only by the assumption that in the former we have a conjugated system of three double bonds or a benzenoid structure. If this assumption is permissible, further insight is furnished into the allocation of the hydroxyls of strophanthidin. The suggestion is offered that at least two are situated on the same nucleus as the carbonyl group and when complete dehydration occurs a benzenoid structure develops. While the following scheme may permit these transformations it is not certain how compatible it is with previously reported oxidation experiments, especially since the course of the latter has not been fully explained.



On turning to the experiments on the hydrogenation of dianhydrostrophanthidin, a decided contrast was noted. When the latter in acetic acid solution was shaken with an active palladium black and hydrogen, 4 mols of hydrogen were absorbed, the first two fairly rapidly, but the remainder requiring a number of days for completion. From the reaction mixture which apparently contained still other substances two isomers were isolated which analyses have shown to possess the formula $C_{23}H_{34}O_3$. Contrary to dianhydrostrophanthidin, the substance obtained in larger amount did not react with ketone reagents and did not form a benzoate. The conclusion seems justified that here again an oxidic structure was present and that during the hydrogenation removal of water had also occurred. The exact mechanism of the reaction is not clear. Acetic acid, when allowed to act upon dianhydrostrophanthidin, was without effect, so that this was not directly responsible for the loss of water. It is possible that the ketonic group was first reduced to the alcohol which then yielded an oxidic structure by sharing the loss of water with the γ -hydroxyl group. The remaining 3 mols of hydrogen were used for hydrogenation of the three olefinic linkings of dianhydrostrophanthidin. The numerous possibilities for the formation of stereoisomers explain the complex character of the reaction mixture. The two isomers which were isolated have been designated as α - and β -octahydrotrianhydrostrophanthidin.

Dihydrodianhydrostrophanthidin on hydrogenation absorbed 3 mols of hydrogen, and apparently identical substances were obtained although in different proportions.

When the hydrogenation of dianhydrostrophanthidin was

stopped after the consumption of 3 mols of hydrogen, again a mixture was obtained from which two substances were isolated which proved to be isomers with the formula $C_{23}H_{32}O_3$. Here again loss of water has occurred, which forces the conclusion that they are likewise derivatives of trianhydrostrophanthidin; viz., α - and β -hexahydrotrianhydrostrophanthidin, respectively.

Experiments were also made which were interrupted at the 1 and 2 mol stage of hydrogenation, and substances were isolated which from analysis were derivatives of the formulas, $C_{23}H_{30}O_4$ and $C_{23}H_{32}O_4$, or dihydrodianhydrostrophanthidin and tetrahydrodianhydrostrophanthidin, respectively. These were, however, obvious mixtures of isomers, the separation of which proved an unprofitable undertaking.

The smooth course of the dehydration of dianhydrostrophanthidin by hydrochloric acid caused us to turn again to a study of the effect of this reagent on strophanthidin itself. Under the special conditions given in the experimental part strophanthidin is in part converted into a crystalline isomer, pseudostrophanthidin, $C_{23}H_{32}O_6$. Contrary to both the parent substance and isostrophanthidin the new compound does not react with ketone reagents. Attempts to prepare other crystalline derivatives have proven thus far unsuccessful. Its relationship to strophanthidin is, therefore, for the moment, obscure. No trianhydrostrophanthidin could be isolated from the reaction mixture. On the contrary, when monoanhydrostrophanthidin was dissolved in concentrated hydrochloric acid it was in part converted into trianhydrostrophanthidin, but the reaction which occurred was obviously more complicated than in the case of dianhydrostrophanthidin. In this respect monoanhydrostrophanthidin occupies an intermediate position between strophanthidin and dianhydrostrophanthidin.

EXPERIMENTAL.

Trianhydrostrophanthidin.—15 gm. of dianhydrostrophanthidin were treated with 225 cc. of concentrated hydrochloric acid (sp. gr. 1.18). By stirring constantly at room temperature for about 30 minutes all was dissolved, forming an olive solution from which, if allowed to stand too long, a resinous deposit began to separate. The solution was then poured into a large volume of

water, causing the formation of a voluminous, amorphous precipitate. This was collected with water and recrystallized from 95 per cent alcohol, giving 11.6 gm. of flat, tapering needles. After repeated recrystallization from alcohol the substance melted at 135.5–137.5°. It is very soluble in benzene, chloroform, acetone, glacial acetic acid, and less readily in cold ethyl alcohol, ether, and ligroin. It dissolves in concentrated sulfuric acid with a light brown color which changes to a deep cherry-red.

$$[\alpha]_D^{21} = +98^\circ \text{ (} c = 1.015 \text{ in dry chloroform).}$$

$C_{22}H_{24}O_2$. Calculated. C 78.81, H 7.48.

Found (a). " 78.50, " 7.64.

(b). " 78.69, " 7.50.

Attempts to form an oxime by the usual method and to benzoylate in pyridine solution resulted only in the recovery of unchanged trianhydrostrophanthidin. It seems, therefore, justifiable to conclude that dehydration of the oxidic form of dianhydrostrophanthidin has occurred, and that a substance has been formed, still retaining the oxidic structure.

The same substance was obtained under similar conditions directly from monoanhydrostrophanthidin although the course of the reaction was more complicated.

2 gm. of monoanhydrostrophanthidin were shaken in 20 cc. of concentrated hydrochloric acid at ordinary temperature. After 45 minutes the starting material had practically all dissolved, but amorphous material had already begun to separate. The filtrate when poured into water gave 1 gm. of a voluminous precipitate. The dilute acid mother liquor on standing yielded small amounts of crystals which formed from alcohol needles and platelets which melted at 200–205°. Because of the small amount of this material it was not investigated further. The main product of the reaction was recrystallized from alcohol and formed needles and plates which were obviously impure trianhydrostrophanthidin. When recrystallized from a small volume of benzene, a less soluble by-product separated as characteristic twin triangular platelets which melted at 210–212°, also in an amount too small for profitable study. The benzene mother liquor on concentration gave a residue which after repeated

recrystallization from alcohol gave characteristic spears which melted at 136–137° and gave no depression when mixed with trianhydrostrophanthidin.

$$[\alpha]_D^{21} = +99^\circ.$$

Found. C 78.44, H 7.53.

Dihydrotrianhydrostrophanthidin.—1 gm. of trianhydrostrophanthidin, which had been repeatedly recrystallized from alcohol, was hydrogenated by dissolving in a small volume of glacial acetic acid and shaking in purified hydrogen with 0.4 gm. of an active palladium black. An active catalyst is essential to the success of this hydrogenation. Some preparations of catalysts which hydrogenated dianhydrostrophanthidin readily, as described later on, did not cause the hydrogenation of trianhydrostrophanthidin.² Absorption occurred at the rate of 8 to 10 cc. per hour for several hours and then gradually decreased. After about 20 hours 65 cc. were absorbed. The theoretical amount for 1 mol of hydrogen is 69 cc. No further absorption of hydrogen took place on reactivation of the catalyst by shaking with air or on addition of more catalyst.

After filtering off the palladium, the acetic acid solution was poured into 2 liters of water. 0.9 gm. of rhombic scales separated. When recrystallized from a small volume of 95 per cent alcohol, the substance formed rhombic and irregularly hexagonal plates, which melted at 132–133°. It is very soluble in benzene, chloroform, acetone, and glacial acetic acid, and moderately soluble in cold alcohol, ether, and hot ligroin. It dissolves in concentrated sulfuric acid with a red-brown color which deepens on standing.

$$[\alpha]_D^{21} = +97^\circ \text{ (c = 1.015 in dry chloroform).}$$

$C_{23}H_{28}O_4$. Calculated. C 78.38, H 8.02.

Found (a). " 78.48, " 8.09.

(b). " 78.24, " 8.04.

Although in general the properties of the substance bear close resemblance to those of dianhydrostrophanthidin itself, a mixture

² The difficulty attending the successful hydrogenation of this substance is similar to that which we have experienced in the hydrogenation of strophanthidin itself. This experience is borne out by the unsuccessful attempts of Thoms and Unger (Thoms, H., and Unger, F., *Z. angew. Chem.*, 1924, xxxvii, 723) to hydrogenate the latter.

of the two substances melted at 110–115°. Dihydrotrianhydrostrophanthidin was also prepared from dihydrostrophanthidin through the intermediate stage dianhydrodihydrostrophanthidin.* 1 gm. of dianhydrodihydrostrophanthidin was treated with 15 cc. of concentrated hydrochloric acid at room temperature and after stirring until solution was complete the mixture was poured into water. The flocculent precipitate was collected with water and recrystallized from 95 per cent alcohol. Characteristic rhombic and hexagonal plates were formed, which agreed in all properties with the above dihydrotrianhydrostrophanthidin.

$$[\alpha]_D^{22} = +96^\circ \text{ (} c = 1.025 \text{ in dry chloroform).}$$

Found. C 78.46, H 8.36.

Hexahydrotrianhydrostrophanthidin.—4 gm. of dianhydrostrophanthidin, which had been repeatedly recrystallized from alcohol, were dissolved in 200 gm. of glacial acetic acid and hydrogenated by shaking with 0.4 gm. of palladium black. Absorption of hydrogen occurred regularly at the rate of 50 cc. per hour during the introduction of the first and part of the second mol of hydrogen and then slowly decreased and after about 5 days reached the rate of 0.5 cc. per hour, when the hydrogenation was discontinued. 787 cc. of hydrogen were absorbed. The theoretical amount for 3 mols of hydrogen is 780 cc.

After filtering off the catalyst, the acetic acid solution was poured into 1.5 liters of water. 3.5 gm. of crystalline material gradually separated which proved to be a mixture of at least two isomers. By repeated fractional crystallization from alcohol their separation was accomplished. The α -isomer obtained from the first fractions, after repeated recrystallization from alcohol, formed irregular leaflets which melted at 183–187°. It is very soluble in chloroform, benzene, acetic acid, ethyl acetate, and acetone, less readily soluble in alcohol and ether, and but slightly so in ligroin. It dissolves in concentrated sulfuric acid with a pale yellow color which develops a strong green fluorescence on standing.

$$[\alpha]_D^{22} = +7^\circ \text{ (} c = 1.340 \text{ in dry chloroform).}$$

$C_{21}H_{31}O_4$. Calculated. C 77.46, H 9.05.
Found. " 77.01, " 9.22.

* Jacobs and Collins,¹ p. 729.

The more soluble β -isomer obtained from mother liquors was also repeatedly recrystallized from alcohol. It forms glistening needles which melt at 224–227°. It is very soluble in benzene, chloroform, and acetic acid, less readily soluble in ethyl acetate, alcohol, and acetone, and but slightly so in ether and ligroin. With concentrated sulfuric acid it gives a pale yellow color and a slowly developing green fluorescence.

$$[\alpha]_D^{25} = +37^\circ \quad (c = 0.500 \text{ in dry chloroform}).$$

$C_{22}H_{32}O_3$.	Calculated.	C 77.46,	H 9.05.
	Found.	" 77.55,	" 9.17.

Octahydrotrianhydrostrophanthidin.—4 gm. of dianhydrostrophanthidin dissolved in 200 gm. of glacial acetic acid were hydrogenated with 1 gm. of palladium black. In a typical experiment the first mol was absorbed within 30 minutes, the second mol after an additional 3 hours, and the rate steadily continued to decrease until at the end of 2 days absorption had stopped. The rate of absorption was found to depend on the character of the catalyst used. The total volume of hydrogen absorbed was 1,050 cc., or exactly the theoretical amount for 4 mols. When poured into 2 liters of water, 3.6 gm. of crystalline material separated from the originally turbid solution. This proved to be a mixture of isomers from which two substances were separated which will be called the α and β forms, respectively. After repeated recrystallization from ethyl acetate or alcohol, 0.8 gm. of flat, 4-sided needles was obtained, the specific rotation of which was not altered by further recrystallization. This substance, α -octahydrotrianhydrostrophanthidin, melted at 231–233°. However, by further recrystallizations, the melting point was raised to 239–242°. This variation in melting point was caused either by the adherence of small amounts of contaminating material or was due to polymorphism. The substance is very soluble in chloroform, benzene, and glacial acetic acid, moderately so in alcohol, acetone, and ethyl acetate, and but slightly soluble in ether and ligroin. The purest preparations dissolved in concentrated sulfuric acid with a very pale yellow color which deepened on long standing to an orange which showed a slight green fluorescence. Less pure samples gave, almost at once, a strong green fluorescence.

$[\alpha]_D^{25} = +49^\circ$ ($c = 1.020$ in dry chloroform).			
$C_{23}H_{24}O_4$.	Calculated.	C 77.03,	H 9.56.
	Found (a).	" 76.82,	" 9.43.
	(b).	" 76.54,	" 9.40.

From the mixture of substances contained in the mother liquors there was obtained in small amount, a second isomer, β -octahydrotrianhydrostrophanthidin, which after several recrystallizations from alcohol and finally from acetone, melted constantly at $210-213^\circ$. From alcohol or acetone it formed large rectangular plates, but from ethyl acetate flat needles separated which were indistinguishable from those of the α compound. The β compound is very soluble in benzene, chloroform, and glacial acetic acid, moderately soluble in alcohol, acetone, and ethyl acetate, and less so in ether and ligroin. In concentrated sulfuric acid it gives the same color reaction as the α -isomer.

$[\alpha]_D^{25} = +67^\circ$ ($c = 1.025$ in dry chloroform).			
$C_{23}H_{24}O_4$.	Calculated.	C 77.03,	H 9.56.
	Found.	" 76.56,	" 9.52.

Attempts to prepare an oxime and a benzoate of the α compound resulted only in the recovery of unchanged material. With the hope that the hydrogenation would be made simpler by starting with dianhydrodihydrostrophanthidin in which the refractory double bond of strophanthidin was disposed of, the hydrogenation of this substance was attempted.

When 1 gm. of dianhydrodihydrostrophanthidin was hydrogenated in glacial acetic acid with 0.2 gm. of palladium black, the theoretical volume for 3 mols of hydrogen (195 cc.) was absorbed. The reaction took about 5 days and the absorption of the last mol of hydrogen was very slow. The crystalline material which was obtained by pouring the solution into water yielded, on fractional crystallization, small amounts of two substances, which, according to the criteria of melting points, rotations, and physical properties, were identical, respectively, with α - and β -octahydrotrianhydrostrophanthidin. However, contrary to the results obtained by starting with dianhydrostrophanthidin in which the α -isomer preponderated, in this case the β form with $[\alpha] = +64^\circ$ was the main product.

Pseudostrophanthidin.—On treating 43 gm. of strophanthidin

with 400 cc. of concentrated hydrochloric acid (1.19) at 0°, it gradually dissolved, forming a yellowish solution which deepened to an olive. After standing for 4 hours at 0°, the solution, from which small amounts of resinous material had begun to separate, was poured into 3 liters of water. The amorphous white precipitate was filtered off at once. The filtrate on standing and rubbing gradually deposited crystalline material, the separation of which continued for several days. More of this substance which was carried down by the above amorphous precipitate was obtained by carefully redissolving the latter in cold concentrated hydrochloric acid and immediately throwing into water. The filtrate deposited additional crystalline substance on standing. When recrystallized from 50 per cent alcohol it formed bundles of needles which when air-dried softened between 123° and 127° and slowly frothed upon further heating. The yield was 10.5 gm. It is very soluble in alcohol and acetic acid, less readily in acetone and chloroform, and very sparingly soluble in ether, benzene, and ligroin. It dissolves in concentrated sulfuric acid with a red-brown color like that given by strophanthidin. It gives a slowly developing green color in the Liebermann cholesterol test. The substance is halogen-free.

$$[\alpha]_D^{21} = +51^\circ \text{ (} c = 1.003 \text{ in alcohol).}$$

Air-Dry Substance. Dried at 100° and 15 mm. over H₂SO₄.

C₂₃H₃₂O₆ · 1½ H₂O. Calculated. H₂O 6.26.

Found (a). " 5.92.

(b). " 5.63.

Anhydrous Substance.

C₂₃H₃₂O₆. Calculated. C 68.27, H 7.98.

Found (a). " 67.71, " 8.04.

(b). " 68.46, " 8.19.

0.1999 gm. of anhydrous substance was boiled with excess of 0.1 N NaOH and titrated back with 0.1 N HCl against phenolphthalein.

Calculated for C₂₃H₃₂O₆, 1 equivalent 4.95 cc. NaOH.

Found. 5.2 " "

Contrary to strophanthidin, during treatment with alkali, isomerization analogous to the formation of isostrophanthidin does not occur. 1 gm. of pseudostrophanthidin was saponified under the conditions used above for titration. The solution was made slightly acid and then concentrated to small volume. On acidifying to Congo red with HCl, an amorphous precipitate formed

which, when recrystallized from 50 per cent alcohol, yielded 0.23 gm. of pointed rods which proved to be unchanged pseudostrophanthidin.

Attempts to form an oxime or a phenylhydrazone of pseudostrophanthidin resulted only in the recovery of unchanged material, a result which demonstrates the absence of a carbonyl group. Although in typical acylation experiments a reaction was observed, the resulting products showed little tendency to crystallize.

All attempts to crystallize the amorphous material which preponderated in the reaction of hydrochloric acid on strophanthidin were unsuccessful, and no evidence of the formation of trianhydrostrophanthidin could be obtained.

A NEW SET OF BUFFER MIXTURES THAT CAN BE PREPARED WITHOUT THE USE OF STANDARDIZED ACID OR BASE.

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The present paper describes a set of buffer mixtures that can be prepared entirely from weighed salts and acids, so that the use of standardized acid or base is not necessary. The standard substances must obey the condition that they can easily be obtained pure. This is the case with succinic acid, acid potassium phosphate, and borax. Mixtures of succinic acid and borax solutions furnish buffers over a range of pH from 3.0 to 5.8, while mixtures of primary phosphate and borax give buffers over a range of pH from 6.0 to 9.2. For physiological work this range of pH (from 3.0 to 9.2) will usually suffice.

Preparation and Purity of the Substances.

Succinic acid can be purified by recrystallization of a high grade commercial sample two or three times from distilled water. It should be air-dried in thin layers, and the constancy of weight established in a desiccator over calcium chloride. It contains no water of crystallization. The succinic acid must not be dried at high temperatures, as 2 molecules of the acid lose 1 molecule of water in forming the anhydride. A stock solution cannot be preserved for a long time, as it soon becomes mouldy. The addition of a little thymol acts as a preservative and is advantageous.

Acid potassium phosphate, a high grade commercial sample, is recrystallized two or three times from distilled water and dried to constant weight at 110–115°. The salt must dissolve clear in water, and its solution (1:20) yield no test for chloride or sulfate. When dried at 110–120° the loss in weight should be less than 0.1

per cent, and on ignition the loss should be 13.23 ± 0.1 per cent. The 0.1 molecular solution should be distinctly red with methyl red, and it should give a yellow-green color with brom-cresol green.

Borax, a high grade commercial product, that is generally pure enough, is recrystallized from distilled water, and constancy of weight established in a desiccator over $\text{NaBr} \cdot 2\text{H}_2\text{O}$ in contact with its saturated solution. In this way the sodium tetraborate with

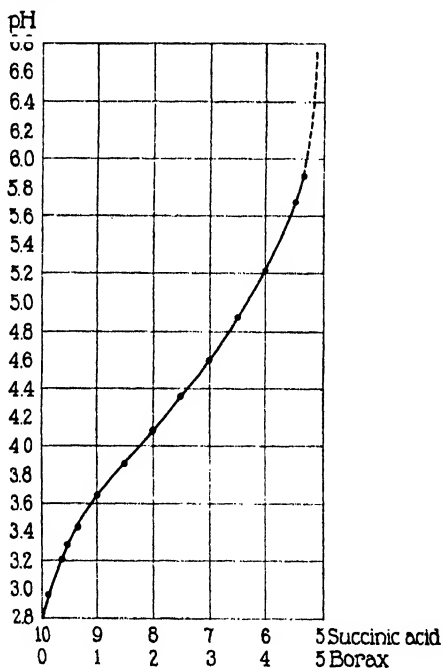


FIG. 1. Succinic acid-borax mixtures.

10 molecules of water of crystallization is obtained; it is a convenient standard for the acidimetry and can be checked against standardized acid with methyl orange or methyl red as an indicator.

The stock solutions for the preparation of the standards are: (a) 0.05 molecular succinic acid (with a trace of thymol), containing 5.90 gm. of acid in 1 liter of solution; (b) 0.1 molecular solution of primary potassium phosphate, containing 13.61 gm. in 1 liter of

solution; and (c) 0.05 molecular solution of borax, containing 19.10 gm. in 1 liter of solution.

Mixtures of the succinic acid and borax solutions and of the acid phosphate and borax solution were prepared, and the hydrogen ion exponent was determined with the hydrogen electrode at 18°. The measurements were carried out in the Physiological Laboratory of the University of Utrecht.

In Figs. 1 and 2 the pH values obtained are plotted as ordinates against the composition of the mixtures as abscissas.

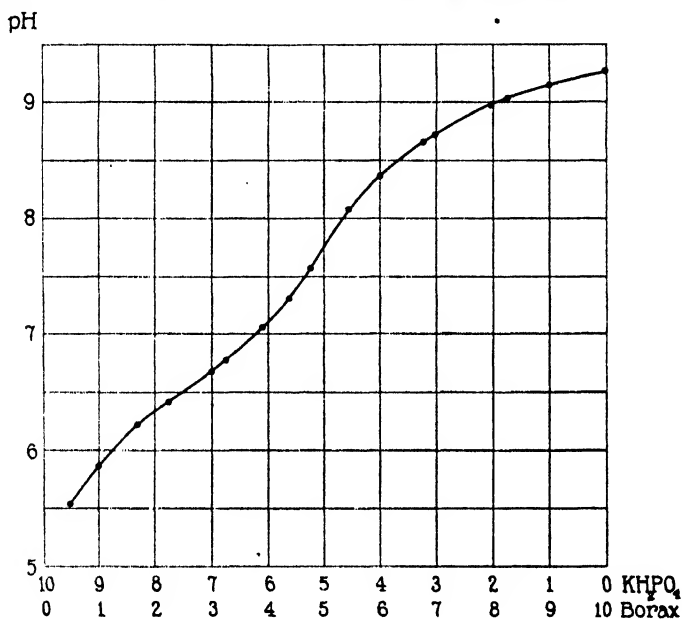


FIG. 2. KH_2PO_4 -borax mixtures.

The figures given show the composition of a total volume of 10. From the graphs we can read the composition of the buffer mixtures corresponding to a definite pH. In Table I the data for the preparation of a set of mixtures that differ by increments of 0.2 pH over a range from pH 3.0 to 9.2 are recorded.

The pH of these mixtures increases appreciably when the solution is diluted. This is shown in Table II where the pH of an undiluted and a tenfold diluted solution is given.

TABLE I.
Composition of Mixtures Giving pH Values at 18°.

0.05 molar succinic acid + 0.05 molar borax mixtures.		
Succinic acid.	Borax.	pH
cc.	cc.	
9.86	0.14	3.0
9.65	0.35	3.2
9.40	0.60	3.4
9.05	0.95	3.6
8.63	1.37	3.8
8.22	1.78	4.0
7.78	2.22	4.2
7.38	2.62	4.4
7.00	3.00	4.6
6.65	3.35	4.8
6.32	3.68	5.0
6.05	3.95	5.2
5.79	4.21	5.4
5.57	4.43	5.6
5.40	4.60	5.8
0.1 molar KH_2PO_4 + 0.05 molar borax.		
KH_2PO_4	Borax.	pH
cc.	cc.	
9.21	0.79	5.8
8.77	1.23	6.0
8.30	1.70	6.2
7.78	2.22	6.4
7.22	2.78	6.6
6.67	3.33	6.8
6.23	3.77	7.0
5.81	4.19	7.2
5.50	4.50	7.4
5.17	4.83	7.6
4.92	5.08	7.8
4.65	5.35	8.0
4.30	5.70	8.2
3.87	6.13	8.4
3.40	6.60	8.6
2.76	7.24	8.8
1.75	8.25	9.0
0.50	9.50	9.2

DISCUSSION.

As has been stated, the great advantage of our buffer standards is, that they can be easily prepared without the use of standardized acid and base. We must, however, also be certain that the intens-

TABLE II.

Composition of the mixture.	pH of undiluted solution.	pH of tenfold dilution.	Δ pH
8.73 cc. KH_2PO_4 and 1.27 cc. borax.	6.035	6.232	+0.20
6.75 " " " 3.25 " "	6.765	7.006	+0.24
5.22 " " " 4.78 " "	7.549	7.688	+0.14
4.57 " " " 5.43 " "	8.076	8.235	+0.16

ity of their buffer action is large enough to assure reliable results. According to Van Slyke,¹ we can express the quantitative relationship of buffer effect to the pH of the solution. The unit of buffer

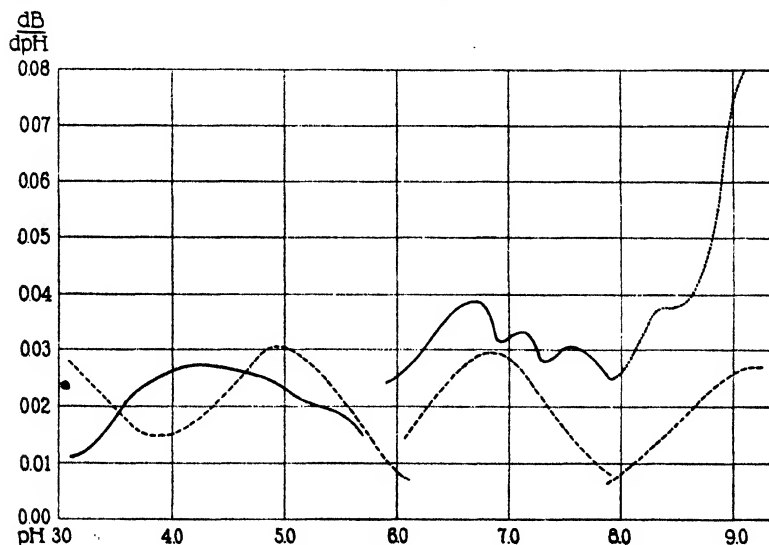


FIG. 3. Buffer capacity of $\frac{dB}{dpH}$ of mixtures of Clark and Lubs (broken line) and of a new set of mixtures (solid line).

¹ Van Slyke, D. D., *J. Biol. Chem.*, 1922, lli, 525.

value (the word buffer capacity seems better to me) is the differential coefficient $\frac{dB}{dpH}$, expressing the relationship between the increment (in gram equivalents per liter) of strong base B added to a buffer solution and the resultant increment in pH. Increment of strong acid is equivalent to a negative increment of base, or $-dB$. In these terms, a solution has a buffer value of 1 when a liter will take up 1 gram equivalent of strong acid or base per unit change in pH.² In this way I have calculated the values of the buffer capacity of the mixtures of Clark and Lubs.³ The results obtained are illustrated by the broken line curves in Fig. 3, in which $\frac{dB}{dpH}$ is plotted as ordinates against the corresponding values of pH as abscissas. Similarly, I have calculated the values of the buffer capacity of the new set of buffer mixtures described above. The results are shown in the figure by the solid lines. For dB is calculated the increment of the borax content and not the corresponding amount of strong base. The consequences of this are discussed below.

We see that the latter curves—especially that of the phosphate-borax mixtures—have not such a regular shape as those of the mixtures of Clark and Lubs. This difference may be explained by the fact that my buffer mixtures were made up in such a way that the total volume remained constant, and the data had to be recalculated for a constant amount of one of the components. So if we express all terms from the succinic acid-borax mixtures on a volume of 10 cc. of succinic acid, we must add 0.142 cc. of borax to obtain a pH of 3.0 and 5.82 cc. of borax to obtain a pH of 5.0 (Table I). In this way we have different end-volumes, whereas the mixtures of Clark and Lubs are related in the same amount of standard substance and the same end-volume. The curves give the impression that the buffer capacity of my mixtures at pH from 8.0 to 9.0 is much greater than that of Clark and Lubs. This is not quite true, as I calculated the value $\frac{dB}{dpH}$ on the

² Van Slyke,¹ p. 528.

³ Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1,109, 191. Compare Clark, W. M., *The determination of hydrogen ions*, Baltimore, 2nd edition, 1922.

assumption that dB represents the number of equivalents of borax that causes the change of pH and not the amount of strong base, as is the case in the calculation of the mixtures of Clark and Lubs. So it is evident that the great buffer capacity of my mixtures at pH from 8 to 9 is only apparent; the curve, however, shows clearly that a small error in the ratio between the mixed acid phosphate and borax solution has but a small influence on the pH of the buffer.

SUMMARY.

A new set of buffer mixtures that can be prepared with solutions of weighed crystalline substances is described. The mixtures of 0.05 molecular succinic acid solution and 0.1 molar acid potassium phosphate cover a range of pH from 3.0 to 5.8; those of 0.1 molecular primary potassium phosphate and 0.05 molar borax, from pH 5.8 to 9.2.

I am greatly indebted to Prof. Dr. W. E. Ringer, in whose laboratory all measurements of the hydrogen ion concentration were carried out. I wish also to express my thanks to Miss Grutterinck who made the majority of the measurements described.

MINERAL DEFICIENCIES OF MILK AS SHOWN BY GROWTH AND FERTILITY OF WHITE RATS.*

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(Received for publication, December 24, 1924.)

In the course of an investigation undertaken to determine the biological value of cow's milk subjected to heat treatment for the purpose of sterilization, it was observed that rats fed exclusively on milk, either raw or quickly boiled, seldom reproduced. In those instances where reproduction did take place, the young in general were born late, and only a very small percentage survived the suckling period. A third generation on milk alone was never raised. Growth in the rats on the quickly boiled milk was much better than on the raw milk. A diet which is in every way satisfactory will permit of early multiplication through an endless number of generations. In estimating the nutritive value of any food mixture assumed to be complete it would seem that the production of a fifth generation in as perfect a state of nutrition as that of the first, would be a fair criterion. The general sterility of our first generation milk-fed rats, and the poor quality of the few examples of the second generation which survived, suggested that milk is deficient in substances essential to normal physiological processes.

In order to make sure that the difficulty was inherent in milk and was not due to the fact that the animals could not ingest enough of the essential ingredients in this dilute form to meet

* The data contained in this report are the results of a number of years of study. The work was begun at the University of Wisconsin and we are indebted to the Department of Home Economics of the University for material and cooperation. We are also indebted to Miss Emma Francis and Miss Rosemary Laughlin for their assistance and untiring efforts during the first years of the work.

the needs of pregnancy and lactation, tests were made with whole milk powder. The results here were quite similar to those with milk alone. Growth in the first generation was normal; only a few young of the second generation were raised, and these never reproduced.

The unsatisfactory results with these milk-fed rats led to a long series of attempts, continued over a number of years, to supplement the milk ration. Since this study was preliminary to its application to problems of infant nutrition, at first only those substances were added which might be included in a diet adapted to very young children. Supplementing milk with such substances as cereal diluents, wheat embryo extract, lactose, beef extract, corn-starch, cod liver oil, and later, cystine, and various calcium salts, failed to improve the nutritive value of milk.

The well known fact that milk is poor in iron led to the addition of small amounts of iron citrate. The percentage of viable young and the number of fertile animals were somewhat higher than in the groups fed milk alone, but both were considerably below normal. The third generation never reproduced.

Since the milk used was produced in the Mississippi Valley, where goiter is quite prevalent, it seemed probable that the milk was deficient in iodine. Therefore, iodine alone, and a combination of iron and iodine were tested. In the latter case slightly better results were obtained than with milk and iron. With one group we obtained a fourth generation which grew normally during the first 3 months. From this time on, however, the animals gained very slowly and at the age of 9 months none of the six females in the group had reproduced. Other groups on this mixture failed to reproduce in the first generation.

Although on the milk ration alone, and on milk to which iron and iodine were added, reproduction never took place after the third generation, there was no uniformity in the number and viability of the young in the various generations. Some females did not reproduce, while others did. One litter would be raised, while others would die or would be destroyed by the mother soon after birth. It was impossible to anticipate what would take place or even to draw conclusions from the results obtained. The erratic behavior of these milk-fed rats strongly suggested that

the essential constituents were present in milk, but in insufficient and varying amounts.

The reports of Ruhräh,¹ lauding the value of soy bean-milk mixtures in cases of undernutrition in infants, suggested its trial in these animal experiments. From 7 to 10 gm. of soy bean powder, cooked and added to 1 liter of boiled milk (7 gm. being the proportions suggested by Ruhräh), produced surprising results. Young in the first generation were obtained at 13 weeks. Second and third litters followed in quick succession; these in turn bore young which were successfully-raised. So satisfactory were these results that a combination of milk and autoclaved soy beans with cracked corn and tricalcium phosphate has become our stock ration, assuring an abundance of virile, disease-resistant animals.

To determine whether the potent substance in the soy beans was organic or inorganic, animals were fed milk to which the ash of soy beans was added. All females in the first generation were fertile, first litters being born before the age of 6 months; the litters were normal in number and the individuals were of average size. 76 per cent of these young were successfully suckled, whereas, on milk in the first generation, only 12.5 per cent of the females were fertile up to 6 months; few young were born and none of these lived. In the second generation on milk alone only one of the five females was fertile up to 6 months and none of the five young born in this group was raised. In contrast to this the ten females of the second generation on the soy bean ash-milk mixture produced 67 young, all of which were raised. In the three successive generations on milk and soy bean ash, an average of 6.3 young per female up to 6 months was successfully suckled. The data are summarized in Table I. The conspicuously better results obtained when the soy bean ash was added to milk would seem to indicate that the inorganic constituents of milk are inadequate for reproduction and successful rearing of young.

An analysis of the soy bean ash showed that aluminum and silicon were present. At first, no significance was attached to these findings. Later, milk feeding tests were made with these additions. The results are discussed in a subsequent section.

¹ Ruhräh, J., *J. Am. Med. Assn.*, 1910, liv, 1664.

TABLE I.

Comparison of Fertility and Viability of the Young of Rats on Various Rations.

Diet.	Generation.	No. of females.	Total No. of young to 6 mos.	Total viable young.	Fertility.	No. of young per female.	Remarks.
				per cent	per cent		
Milk.	1	8	8	0	12.5	0	
	2	5	5	0	20	0	
" and iron.	1	3	15	40	100	2	
	2	7	41	34	57	4.3	
	3	3	0	0	0	0	
" iron, and iodine.	1	6	23	47	66	2	Addition of the 4 minerals to the 4th generation at 7 mos. was without effect.
	2	3	28	47	100	2.3	
	3	10	46	0	40.6	0	
	4	5	0	0	0	0	
Milk, starch paste, iron, and iodine.	1	4	12	0	50	0	
Milk and soy bean.	1	3	35	80	100	8.1	Grew well up to 3 mos. when experiment was discontinued.
	2						
" " " " ash.	1	11	86	76	100	6.0	0.364 gm. ash, minimum amount per liter of milk.
	2	10	67	100	100	6.7	
	3	3	20	100	100	6.3	
Stock ration.		4	34	79	100	6.7	
Experimental ration.	1	4	27	100	100	6.5	
	2	4	27	100	100	7	

Osborne and Mendel² have reported significant traces of aluminum, fluorine, and manganese in milk and have used these in

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

their purified rations in the proportions found. Whether these play any vital rôle in animal physiology has not been determined. Certain of these are present in various animal tissues and all are constantly found in plants. It seemed possible that some or all might be essential to physiological processes, and although they are present in cow's milk, they may be in too small concentration for reproduction and rearing of the young of the particular species under investigation. Since silicon is found in all vegetable and in some animal tissues, it was thought that this also might be essential to physiological processes. Accordingly a thick starch paste, containing a mixture of aluminum potassium sulfate ($\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$), sodium fluoride (NaF), sodium silicate (Na_2SiO_3), and manganese sulfate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$), was added to milk in such a proportion that each animal received daily 1.5 mg. of each salt, a purely arbitrary amount. The starch paste was boiled, cooled, and the required amounts of solutions of the various salts were incorporated separately. In all cases the milk used was brought quickly to the boiling point, not more than a pint being heated at one time. 1 drop of a 2 per cent solution of sodium iodide and 3 drops of a saturated solution of iron citrate were added to each 200 cc. of milk. The starch paste mixture was then distributed in small pieces in the milk. The feeding containers were filled with milk twice daily, assuring an abundance of food. All animals were kept in metal-lined cages, with wood shavings for bedding.

On milk supplemented with these four mineral constituents, the rats developed admirably; young were born between the 14th and 16th weeks. These were vigorous and developed normally. In each successive generation, all females were fertile, first litters being produced early and a large proportion of the young, an average of 80 per cent in the five generations, was raised. Six generations have been successfully produced with every indication of continued growth and reproduction. Two females of the sixth generation bore young at $3\frac{1}{2}$ and $3\frac{3}{4}$ months, respectively. From these results it would seem that the substances lacking in milk are included in this salt mixture. It remained to be determined, however, which of the salts, whether all or only part of them, contributed to the excellent results. Trials were therefore made with single salts and combinations of two and three.

The initial animals used in all the experiments were taken at approximately 5 weeks of age (45 to 60 gm.) from the stock group which had been fed our standard ration. The experimental groups, in general, consisted of four females and two males. Occasionally in subsequent generations two or three females only were included in a group, owing to the fact that a small number of young were raised or too few females were produced. Both males and females in the various groups were animals of the same generation receiving the same experimental salt mixture. Frequently they were all of the same litter. In a few instances where the females failed to reproduce after a reasonable time, 7 or 8 months, stock males were put with the females in order to rule out the possible impotency of the males. The pregnant animals were isolated several days before parturition and were so cared for until the young were 4 or 5 weeks old. In a few groups in which all the females failed to reproduce after 7 and 8 months on the given mixture, a combination of the four minerals was used.

In summarizing the results (Table II) we have given the number of females in a group, the total number of young born up to 6 months, the percentage of these raised, the percentage of fertile females, arbitrarily setting the standard of fertility at 6 months, and the average number of viable young per female in the 6 month period. In a number of cases young were born when the animals were older than 6 months, but since late initial pregnancies are an indication of faulty diet, it seemed that 6 months was a fair time allowance. Furthermore, it seemed probable that late pregnancies in our animals might be the result of an accumulation of the substances necessary for reproduction and rearing of young, a certain amount of which apparently is contained in milk. It sometimes happened that young were born and destroyed by the mother before they could be counted. When this occurred we have estimated the litter as six, a fair average for the animals on stock rations.

In the table there may seem to be some discrepancies. Groups are reported in which there are no viable young, yet a next generation has been carried on. In these cases the colony was made up of young born after the females were more than 6 months of age.

TABLE II.

Influence of Certain Inorganic Substances on the Fertility and Viability of the Young of Milk-Fed Rats.

Diet.	Generation.	No. of females.	Total No. of young to 6 mos.	Total viable young.	Fertility.	No. of young per female.	Remarks.
				per cent	per cent		
Aluminum, manganese, fluorine, silicate, and milk.*	1	11	96	97	100	8.5	
	2	9	70	70	100	5.5	
	3	5	36	59	100	4	
	4	4	43	83	100	9	
	5	4	34	91	100	9	
Milk,* aluminum, fluorine, and silicate.	1	6	30	93	100	4.6	
	2	4	28	19	68	4.7	
	3	4	33	60	100	5	
	4	4	23	26	50	1.5	
	5	4	12	83	75	3	
Milk,* aluminum, manganese, and fluorine.	1	5	10	40	40	0.8	
	2	3	13	23	66	1	
	3	5	23	73	60	3.5	
	4	4	17	23	100	1	
	5	4	13	84	50	2.7	
Milk,* aluminum, manganese, and silicate.	1						
	2	4	24	100	75	6	
	3	4	26	42	75	2.75	
	4	4	27	55	75	3.75	
	5	4	0	0	0	0	
Milk,* aluminum, and manganese.	1	4	0	0	0	0	
Milk,* aluminum, and fluorine.	1	3	0	0	0	0	
Milk,* silicate, and fluorine.	1	4	0	0	0	0	
Milk,* aluminum, and silicate.	1	4	36	78	100	7	
	2	4	0	0	0	0	
	3	4	35	11	100	1	
	4	4	19	38	51	1.8	
	5	4	0	0	0	0	

TABLE II—*Concluded.*

Diet.	Generation.	No. of females.	Total No. of young to 6 mos.	Total viable young.	Fertility.	No. of young per female.	Remarks.
				per cent	per cent		
Milk* and manganese.	1	3	3	0	33	0	3 animals in group of 6 died early.
Milk* and fluorine.							Grew poorly. Died at 3 mos.
“ “ alum- inum.	1	4	19	95	100	4.7	Females of 4th generation subsequently bore young when 4 minerals were added at 8th month.
	2	4	22	64	75	3.5	
	3	4	39	51	100	5	
	4	4	0	0	0	0	
Milk* and silicate.	1	4	34	68	75	5.6	
	2	4	19	68	75	3.2	
	3	4	20	70	75	5.3	
	4	4	27	32	100	2.7	
	5	4	13	46	75	1.5	

* Iron and iodine were added.

In evaluating the results of the various salt additions we at first believed that fertility would be the criterion of the efficiency of a given mixture, but the high mortality of the young in the successive generations of many of our experimental groups, even when fertility was high, led us to conclude that the number of young raised would be a better method of determining the potency of the added salts. With a few exceptions, the percentage of fertility in the various groups up to the fourth generation was fairly comparable, and within the limits of expectancy when there were only four females in a group; and although early pregnancies, between 3 and 4 months, are the rule when all conditions are perfect, the slight variations in the fertility of the successive generations up to the 6th month, indicated that fertility alone could not be used as a measure of nutritive efficiency in these

cases. Instead, the average number of young per female, raised within a 6 month period, has been the standard used.

Judging the effects of the various salt additions by this standard it is readily seen that those animals receiving the mixture of four salts outclassed all the other groups. The number of viable young in each generation compares favorably with that obtained with our stock animals and with two generations on an experimental ration which we had every reason to believe contained the essential ingredients of a diet in adequate amounts. In order to make sure that the results with this salt mixture were not due to individual variations, or environmental conditions which we might not appreciate, several groups have been tested from time to time. The response in each case has been the same, early reproduction and normal young.

In only one group on this mixture have we had a high percentage of infant mortality. In this, all the females were fertile, and a normal number of young were born. Some of the litters, however, were destroyed by the mothers soon after birth. But since litters of four, five, and eight, respectively, have been raised in this group, we attribute the cannibalistic tendencies of some of these mothers to nervousness and fear, for during this period construction work was going on outside the animal room, producing much noise and confusion.

The results obtained with the single salt additions, and the combinations of two and three, are difficult to interpret. The addition of aluminum ($\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$) in one series, and of sodium silicate (Na_2SiO_3) in another, resulted in a high percentage of fertile animals and a fair number of viable young through three generations. When these two salts were used together, all females in the first and third generations were fertile, whereas those in the second and fifth reproduced very late. With the exception of the first, the number of viable young in each generation was very low. The addition of either manganese or fluorine to the aluminum and silicate mixture resulted in a high percentage of fertility and a fair number of viable young. Whether manganese or fluorine are essential is not clear. Alone in the concentrations used, they appeared to be toxic. Combinations of aluminum and manganese, and aluminum and fluorine, were also unsuccessful. In neither case were there young in the first

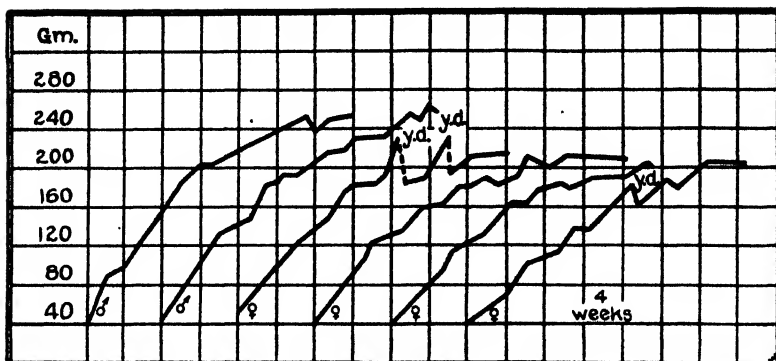


CHART 1. Rats fed milk supplemented with iron and iodine grew well. These seldom reproduced and the young died soon after birth. All conditions in these two groups of animals (Charts 1 and 2) were the same with the exception noted. They were fed at the same time of year, conditions of housing, bedding, etc. were identical, and the milk was obtained from the same source.

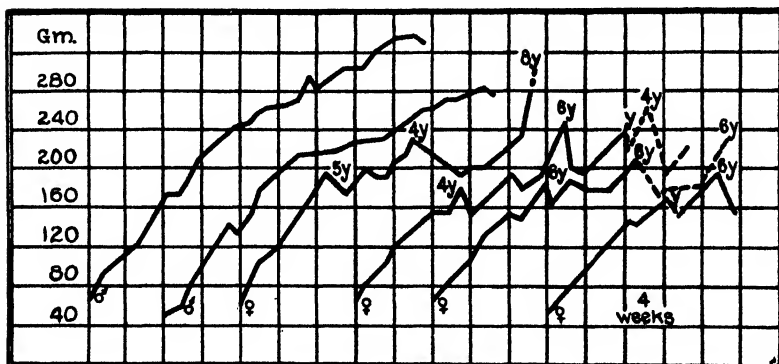


CHART 2. Milk-fed rats which were receiving, besides iron and iodine, 1.5 mg. of aluminum potassium sulfate, sodium fluorine, sodium silicate, and manganese sulfate per rat, per day, grew normally, reproduced at frequent intervals, and raised a large proportion of their young.

generation. A combination of aluminum, manganese, and fluorine, however, carried on to the fifth generation. With the exception of the fourth generation, fertility in these was slightly

below normal; and the number of viable young was considerably below the average in each generation.

Since these experiments have been conducted throughout a number of years, it seemed possible that some of the apparent inconsistencies might be attributed to seasonable variations. Tabulations of the number of young born in the several months, as well as the productive age of the females in relation to time of year, did not indicate that variations in the milk or season were concerned with these results. Nor can we attribute the variations to unusual substances contained in either the starch or shavings, used for bedding, for each group was similarly housed and all received the starch paste. Charts 1 and 2 illustrate the marked difference in the results obtained with the milk and starch paste, and the milk, starch paste, and four salt mixture, respectively. These two groups were run at the same time and under the same conditions of housing, bedding, etc. The source of milk, starch, iodine, and iron used was the same in each group.

DISCUSSION.

Unsatisfactory growth and reproduction in rats on a diet of milk have been reported by Mattill and Conklin,³ and Mattill and Stone.⁴ These authors believe that milk is lacking quantitatively and qualitatively in substances necessary for adolescent growth and reproduction. Sure,⁵ and Evans and Bishop,⁶ working with milk and synthetic rations made to simulate milk, have also reported failures in the reproductive functions in rats. This they attribute to a dietary deficiency, a vitamin X, which is present in a wide variety of foods, but which is low in milk. According to Mattill, Carman, and Clayton,⁷ both sexes are affected when this substance is quite lacking or in low concentration. Degenerative changes were observed in the gonads of the male, and, although ovulation seemed to be normal in the female, im-

³ Mattill, H. A., and Conklin, R. E., *J. Biol. Chem.*, 1920, xliv, 137.

⁴ Mattill, H. A., and Stone, N. C., *J. Biol. Chem.*, 1923, lv, 443.

⁵ Sure, B., *J. Biol. Chem.*, 1923-24, lviii, 681, 693.

⁶ Evans, H. M., and Bishop, K. S., *J. Am. Med. Assn.*, 1923, lxxxi, 889; *Anat. Rec.*, 1924, xxvii, 203.

⁷ Mattill, H. A., Carman, J. S., and Clayton, M. M., *J. Biol. Chem.*, 1924, lxi, 729.

plantation was unsuccessful. The females regained their procreative capacity soon after the renewal of the vitamin X supply. The males, on the other hand, appeared to be irreparably damaged if deprived of vitamin X beyond the 150th day, or thereabouts, of life.

That all substances necessary for normal growth and reproduction are in milk, but in too small concentration, seems evident, not only from our work but from that of Anderegg,⁸ and Mattill, Carman, and Clayton. Both of these authors have observed that in animals fed milk low in fat marked reproductive failure does not occur. Apparently, when more milk is taken, as would be the case in rats fed a low fat milk, enough of the various essential substances are obtained for the production of a next generation. Since we have obtained five generations of normal young on whole milk (3.25 per cent fat) to which our four mineral mixture has been added, it would seem that the deficiency of milk lies in its inorganic components. Whether all four of the salts used in our studies are in low concentration in milk is not clear, nor from our results can we determine which are most essential. Aluminum and sodium silicate appear to be necessary. These, however, must be so incorporated in a diet that they are both available. It seems probable that the confusing results, obtained in some of these groups which received the single salts and the mixture of two and three, have been due: (1) to the fact that milk contains a varying amount of the necessary substances which, in certain cases, has been enough to carry over to a second generation; and (2) to the fact that chemical interactions in the combinations used, rendered certain of the salts inert or have made just enough of others available to produce the results reported. The number of viable young obtained in those groups fed milk and sodium silicate, and milk and aluminum potassium sulfate, respectively, in contrast with the number obtained when milk, sodium silicate, and aluminum potassium sulfate were fed in combination, may be cited as an example. The happy results produced where manganese and fluorine were added to the aluminum and silicate mixture may have been due to the fact that the aluminum and silicate were thus made more readily available; on the

⁸ Anderegg, L. T., *J. Biol. Chem.*, 1924, lix, 587.

other hand, traces of manganese and fluorine may be necessary for physiological processes.

The observations of Mattill, Carman, and Clayton to the effect that the gonads of the males on the milk diet become degenerate, whereas the females need only to have those essential substances incorporated in the diet to bring about normal reproductive functions, may explain some puzzling results in our work. The introduction of stock males into those groups which had failed to reproduce in 7 and 8 months was without effect. Furthermore, the addition of the mixture of four minerals to the milk-fed rats which had failed to reproduce after 7 months was ineffectual. When, however, the mixture of four minerals was added to the fourth generation of aluminum and milk-fed animals which had failed to reproduce in 7 months, all females in the group subsequently bore young. Failure in the reproductive function in the milk-fed group may be attributed to gonad degeneration caused by a deficiency of one or more essential constituents. With the aluminum and milk-fed rats the deficiency was not such as to bring about irretrievable damage.

Since failure in the reproductive function has been observed in rats fed both milk and a synthetic ration made to simulate milk, it seemed probable that the nutritive deficiency might be the same in each case, and that the potency of vitamin X, postulated by Evans and Bishop, might be due to the presence of minute quantities of inorganic fat complexes in the alcohol-ether extracts used. However, the addition of the four minerals (aluminum, silicon, fluorine, and manganese) to our purified ration in the same concentration in which they were used in the milk feedings was without effect. It would seem, therefore, that if vitamin X is essential to physiological processes, it is contained in milk in sufficient quantity for growth and reproduction. Our purified ration, on the other hand, may be deficient in both X and certain inorganic constituents, or it may be that the amount of the various salts added to our purified ration was insufficient, since no aluminum, silicate, manganese, or fluorine was used in the original synthetic mixture. This remains to be determined.

SUMMARY.

1. Rats fed exclusively on cow's milk seldom reproduce, and only a very small percentage of the young born survive.

2. The nutritive deficiency of milk appears to be due to the fact that milk is low in certain inorganic substances necessary for the production of a new generation.

3. The addition of from 7 to 10 gm. of soy bean powder to 1 liter of milk was effective in correcting this deficiency. The addition of the ash of the soy bean was also effective in correcting the deficiency.

4. The addition to milk of those unusual mineral substances present in milk in low concentration, namely manganese, fluorine, and aluminum, together with sodium silicate have resulted in the production of five generations of normal young.

5. These four salts used alone or in combinations of two and three were less effective in correcting the nutritive deficiencies of milk than was the mixture of four salts.

6. The addition of aluminum, manganese, fluorine, and silicon to a purified ration made to simulate milk, in the same concentration in which they were used in milk, was without effect on the reproductive processes. It is suggested that failure may have been due to a lack of vitamin X in this mixture or to too little of the added salts.

COLORIMETRIC DETERMINATION OF TRYPTOPHANE BY THE VANILLIN-HCl REACTION AND THE QUANTITATIVE SEPARATION FROM INDOLE AND SKATOLE.*

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I.

INTRODUCTION.

A critical review of the published methods for the estimation of tryptophane as such and in proteins reveals the need of a better method. A number of the early colorimetric methods are summarized very briefly by Herzfeld (1). From our studies in general, we conclude that not one of the color reactions used is specific for tryptophane in the final determination in that tryptophane decomposition products as well as some other products of protein hydrolysis give identical or different colors with the respective reagents. We therefore felt the need of a specific quantitative method for tryptophane itself.

Various aldehydes giving colored condensation products with tryptophane were tried on tryptophane and other amino acids. Of these it was found that vanillin, which appeared to be most sensitive with tryptophane, gave no color with tyrosine, cystine, alanine, phenylalanine, histidine, glucosamine, or leucine. Steensma (2), in his work on color reactions with proteins, indole and skatole, used 5 per cent vanillin in 95 per cent alcohol and 0.5 per cent NaNO_2 in water. He reported that proteins boiled with 25 per cent HCl and sufficient of this reagent give a red color without, and a blue color with NaNO_2 . Indole gives an orange

* I wish to acknowledge the help and criticism of Professor F. C. Koch throughout this work.

color, skatole a red to a violet without, and blue-violet with NaNO_2 . Blumenthal (3), working on a method of detecting indole in the presence of skatole, used vanillin as above, but found higher concentrations of vanillin to yield better results. He used a 10 per cent alcoholic solution of vanillin and reports that indole can be detected in concentrations of 1:5,000,000 and skatole in 1:1,000,000, but he was not successful in determining indole in the presence of skatole. He found both colored products to be soluble in amyl alcohol. We found the tryptophane-vanillin color also somewhat soluble in amyl alcohol. It was evident at once that only the strictest quantitative procedure would be of any value and that the reaction could not lend itself to the direct determination of tryptophane in the presence of indole and skatole. Since indole and skatole are biologically important decomposition products of tryptophane, it was obvious that a quantitative separation was essential if the reaction was to be applied to quantitative biological studies involving tryptophane, indole, and skatole.

II.

Quantitative Development of the Method and Its Application Directly on Pure Tryptophane and Indirectly on the Mercury-Tryptophane Compound.

(A) *Color Reaction of Vanillin with Tryptophane.*—A systematic study of the color reaction of vanillin with tryptophane in the presence of acid brought out the following facts.

1. Alcoholic solutions of vanillin are not stable, in that they discolor on standing. Such alcoholic solutions even when freshly prepared develop some color when treated with concentrated HCl. Solutions of vanillin in 50 per cent acetic acid are stable and do not develop this color with strong acids. Furthermore, acetic acid does not interfere with the tryptophane-vanillin color formation.

2. Varied concentrations of different acids were tried and although the characteristic color can be obtained with 50 per cent H_2SO_4 , concentrated HCl was found to be more reliable as a condensing agent between tryptophane and vanillin. A series of experiments carried out with varying amounts of concentrated HCl showed that 15 cc. are the optimum minimum for a 50 cc.

volume; *i.e.*, 5 or 10 cc. are not sufficient for complete color development and 20, 25, or 50 cc. can be used. Since the reaction is neither aided nor retarded by the use of more than 15 cc. of concentrated HCl and inasmuch as it is not very pleasant to make colorimetric readings with concentrated HCl, the optimum minimum, or 15 cc. for a 50 cc. volume, was used throughout the work.

3. The water content must be kept very low during the color development. A better color is obtained by using 0.5 cc. of a 0.05 per cent tryptophane solution than by using 5 cc. of a 0.005 per cent tryptophane solution.

4. This led us to try various condensing agents, such as ZnCl_2 and AlCl_3 , which were put directly into the concentrated HCl with the intent of dehydrating the acid and making the reaction more sensitive. No perceptible improvement was noted, hence their use was abandoned.

5. Oxidizing agents were found to hasten the reactions involved, but a slight excess has a destructive effect. This confirms the work of Mottram (4) on the glyoxylic acid reaction.

6. The concentration of vanillin is very important in determining the intensity and speed of the development of the color with tryptophane. Quantitative color intensities are obtained when the molecular ratio of vanillin to tryptophane is roughly 6 to 1. A constant concentration of vanillin can be used with concentrations of tryptophane varying from 0.2 to 1 mg., and 1 mg. in a 50 cc. volume gives too intense a color for accurate colorimetric comparisons.

7. The optimum time for the maximum color to develop was observed. The results show a good color in 24 hours with a maximum in 48 hours, remaining constant for 120 hours, and then gradually fading.

8. The effect of temperature upon the reaction is very similar to that of oxidizing agents; *i.e.*, it hastens the color formation, but it has a destructive effect and also introduces a secondary reaction, giving the color a reddish tint which makes colorimetric comparison impossible.

(B) *Procedure without Mercury Precipitation.*—The optimum conditions for the vanillin-tryptophane color formation as found from the above studies are obtained by introducing into a 50 cc.

TABLE I.

Tube No.....	1	2	3	4	5	6	7	8	9	10
0.05 per cent tryptophane, cc.....	0.5	0.5	0.7	0.8	1.0	0.5	0.7	0.8	1.0	1.0
50 per cent H ₂ SO ₄ , cc.....	0	1	1	1	1	1	1	1	1	0
HgSO ₄ reagent, cc.....	0	3	3	3	3	3	3	3	3	0
5 per cent H ₂ SO ₄ to volume, cc.....	0	4.5	4.3	4.2	4.0	4.5	4.3	4.2	4.0	0

Vanillin-HCl reaction as indicated above.										
Readings.....	27	24	17.2	15	11.9	23.8	17.1	15	12	14.9
Calculated readings.....	26.8 (24) (?)	23.9 (24)	17.0 (17.1)		12.0 (12)	23.9 (24)	17.2 (17.1)	15.1 (15)	12 (12)	15 (12)

volumetric flask between 0.2 and 1 mg. of tryptophane in 2 cc. of water, next adding 0.4 cc. of 0.5 per cent vanillin in 50 per cent acetic acid, mixing, and then adding 15 cc. of concentrated HCl. After standing for 24 hours, the mixture is diluted to 50 cc. with water, mixed well, and the color compared in a colorimeter within several hours.

A series of comparative tests was carried out directly with varying amounts of pure tryptophane without mercuric sulfate precipitation, and it was found that the tryptophane could be recovered quantitatively.

(C) *Procedure with Mercury Precipitation.*—Comparative tests were next carried out on tryptophane after the separation of its mercury compound as follows:

Into 15 cc. centrifuge tubes, labelled 1 to 10, inclusive, varying amounts of tryptophane were measured. Tubes 2 to 9, inclusive, were treated with the HgSO_4 reagent.¹ The precipitate was allowed to stand for 2 hours, then centrifuged, and decanted. The precipitates were suspended in 0.4 cc. of the vanillin solution and on adding 0.5 to 1 cc. of concentrated HCl the precipitates dissolved. The solutions were then rinsed into 50 cc. volumetric flasks by using small portions at a time of the 15 cc. of concentrated HCl necessary for the reaction. Tubes 1 and 10 were treated directly with the vanillin-HCl reagent as in (B) above, and compared with the standard tube (No. 4, Table I), to note whether mercury precipitation affected the tryptophane color.

Results.

This experiment shows: first, that varying amounts of pure tryptophane are recovered quantitatively by the acid-mercury treatment shown in Tubes 2 to 9, inclusive; and secondly, that the mercury precipitation intensifies the color formation as shown in Tubes 1 and 10, both of which have not gone through the mercury precipitation and which read about 3 mm. too high. Therefore, if the mercury precipitation is used the standard must also be treated in the same manner.

Since the mercury-tryptophane compound gave a more intense color than tryptophane itself, as pointed out above, it was desirable to see whether the effect of the Hg in the mercury-tryptophane

¹ C. W. Austin's unpublished findings from this laboratory as to the optimum conditions for the complete precipitation of tryptophane were followed.

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compound was maximum. It was found that a further addition of HgSO_4 to the mercury-tryptophane compound intensified the color still more. Hence the effect of adding the HgSO_4 reagent to the precipitated mercury-tryptophane compound was studied and it was found that the optimum maximum for the concentrations with which we are working is 1 cc. of 2 per cent HgSO_4 solution in 5 per cent H_2SO_4 . The reaction, therefore, was carried out as described in the first paragraph of (C) above, but adding in addition 1 cc. of 2 per cent HgSO_4 to the mercury-tryptophane precipitate after it was washed, centrifuged, and decanted.

(D) *Application of the Method to Solutions of Pure Tryptophane.*—Definite amounts of a 0.5 per cent tryptophane solution were accurately measured out by an instructor into 25 cc. volumetric flasks, using the same pipettes as were used in the work through-

TABLE II.

Solution No.	Direct (duplicates).		Indirect (duplicates).		Amounts measured off.
1	9.58	9.47	9.61	Same.	9.50
2	14.00	13.82	13.98	14.01	14.00
3	8.07	8.09	8.04	8.09	8.00
4	22.73	22.62	22.73	Same.	23.00(?)
5	20.13	20.22	19.90	20.03	20.00
6	29.13	29.00	29.10	28.76	29.00

out. Also, a standard was made from the same solution so that 1 cc. of the standard contained 0.25 mg. of tryptophane. All were made up to volume. The analyses were then carried out by the author.

A preliminary vanillin-HCl reaction was carried out on 2 cc. portions of each of the diluted solutions and compared with 2 cc. of the standard. An approximate idea of the relative concentrations of the solutions was thus obtained and proper dilutions were then made. The direct vanillin-HCl reaction as well as the mercury precipitation were then carried out on the diluted samples, and their results reported as mg. of tryptophane (Table II).

The recovery of tryptophane by the direct as well as by the indirect procedure indicates the applicability of the method for quantitative studies, since the variations between duplicate deter-

minations are of the same general order as between the amounts measured off and the amounts recovered.

III.

Specificity of the Color Formation and the Quantitative Recovery of Tryptophane from a Mixture of Indole and Skatole by Toluene.

With indole the vanillin-HCl reagent gives an orange and with skatole² a purplish red color. With a tryptophane-indole-skatole mixture the red predominates. Indole and skatole are difficultly soluble in water. However, they are sufficiently soluble to make 0.005 M indole and 0.0025 M skatole solutions. These as well as 0.0025 M tryptophane solutions and mixtures of the three were shaken with various organic solvents. It was observed that indole and skatole are very soluble in toluene, and we soon found that these two substances can be separated quantitatively from tryptophane by means of toluene.³

Crucial experiments were conducted in which tryptophane was quantitatively recovered from mixtures containing indole and skatole after the removal of the two latter by several extractions with toluene. Mercury precipitation does not separate the tryptophane from such mixtures.

IV.

Application of the Method to Protein Hydrolysates.

(a) *Hydrolysis by Barium Hydroxide.*—It was next necessary to show that tryptophane can be recovered from a protein hydrolysate. For that purpose a tryptophane-free protein was sought to which a known amount of pure tryptophane was to be added for recovery. Gelatin was first considered. Homer's Ba(OH)₂ hydrolysis was carried out as indicated in Table III, using Nelson's photographic gelatin.

² Skatole was prepared according to Fischer (5).

³ Baker's toluene gave a color with the vanillin-HCl reagent, hence it was found necessary to wash with H₂SO₄, and distill. Portions of such purified toluene gave no color with the vanillin-HCl reagent.

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I, II, and III were introduced into 150 cc. flasks. These were fitted with reflux condensers, plugged with calcium chloride tubes loosely filled with soda-lime and CaO, and then heated on a water bath for 40 hours. A yellow coloration was noted in I and II. The barium was removed by adding H_2SO_4 while the solution was warm until a slight test for SO_4 was obtained and the solution was slightly acid. Each was then filtered into 100 cc. volumetric flasks and made up to volume. I and III were, therefore, equivalent to a 0.05 per cent tryptophane solution.

The vanillin-HCl test was next carried out by the procedure under (B) upon 1 cc. portions of Hydrolysates I, II, and III, and upon an untreated tryptophane standard of 0.05 per cent tryptophane content, and the colorimetric readings were made as usual. It was impossible to make colorimetric comparisons because III had a pink tint, I a reddish tint, and II, which contained hydrolyzed gelatin alone, had a faint flesh color.

TABLE III.

Sample No.	Gelatin.	0.5 per cent tryptophane solution.	Ba(OH) ₂	Water.
	gm.	cc.	gm.	cc.
I	2.5	10	8.75	52.5
II	2.5	0	8.75	52.5
III	0	10	8.75	52.5

TABLE IV.

I	II	III	Standard.
Purple.	Flesh.	18.7 18.8 18.7	15
		Same character of color and good comparison.	

It appeared that the slight color in II was not sufficient to account for the entire variation and since III did not have gelatin in it, but showed a different tint of color, a portion of each solution was shaken with toluene. The toluene phases from I and III in each case gave a reddish orange test with the vanillin-HCl reagent, thus indicating indole or skatole (?). II was negative in this respect.

The vanillin-HCl reagent when applied to the aqueous phases after toluene treatment gave the colors listed in Table IV.

The toluene treatment removed the interfering substances in III completely, but only partially in I. This experiment shows that Ba(OH)₂ hydrolysis of pure tryptophane introduces an error

of almost 20 per cent and that a reactive compound other than tryptophane, indole, or skatole is present in gelatin or is formed during the alkaline hydrolysis. Waterman (6), who tried the Hopkins and Cole method of the direct isolation of tryptophane from the $\text{Ba}(\text{OH})_2$ hydrolysis of protein, also reports that the hydrolysis cannot be carried to completion without destroying some tryptophane.

It was thought that possibly the disturbing factor was of melanoidin character, and that the mercury precipitation of tryptophane at a high acidity would prevent melanoidin substances from coming down. The mercury precipitation was tried on the above hydrolysates directly as well as on the toluene-treated portions, and then the vanillin-HCl reagent was used, but the results were very similar to those in Table IV; *i.e.*, not all of the reddish tint in I was removed, and accurate colorimetric comparisons were not possible.

Gelatin not being a satisfactory protein for our purpose, it was thought that zein might be used instead, because it is generally considered to be tryptophane-free. Zein was prepared according to Osborne and Clapp (7). Zein⁴ thus obtained gave a slight characteristic tryptophane color with the vanillin-HCl reagent. Upon repurification, it still gave the reaction. Since it was our first experience in the preparation of zein, it was thought that perhaps it was contaminated by other proteins from the corn. However, through the kindness of Professor Wells of the Department of Pathology, a small sample of the zein originally prepared by Osborne and Mendel was obtained. This zein, the same as our preparation, gave a similar tryptophane color reaction. It was obvious then that zein could not be used for the purpose intended and that the effect of protein-split products upon the tryptophane method would have to be studied in some other way.

(b) *Hydrolysis by Pancreatin.*—The question of protein hydrolysis without impairing the tryptophane content, however, was still before us. $\text{Ba}(\text{OH})_2$ hydrolysis showed a loss of tryptophane; acid hydrolysis was out of the question as it is well known that

⁴ We wish to express here our thanks to Professor L. Smith of the University of Illinois for supplying us with the high protein corn used for this purpose.

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tryptophane is decomposed by such treatment, therefore, enzyme hydrolysis was tried.

150 cc. Erlenmeyer flasks, labeled I, II, III, and IV, respectively, were prepared as shown in Table V.

The flasks were well shaken, preserved with toluene, and I, III, and IV were incubated at 35°C., while II was kept at room temperature. On the 6th day 3 cc. portions of each flask were

TABLE V.

Flask No.	0.5 per cent tryptophane solution.	Casein.	0.35 per cent Na ₂ CO ₃ solution.	H ₂ O	2 per cent U.S.P. pancre- atin solution.
	cc.	gm.	cc.	cc.	cc.
I	3	0	25	10*	0
II	3	0	0	35	0
III	3	0	25	0	10*
IV	0	1	25	3	10*

* These were added in amounts of 2 cc. on 5 successive days.

TABLE VI.

(Standard) I	II	III	IV
15	15.0 15.1 14.9	14.0 14.0 14.1	17.2 17.1 17.1

TABLE VII.

(Standard) I	II	III	IV
15	15.0 14.9	13.8 13.7 13.8	16.9 17.0 16.9

taken and neutralized by adding a drop or two of 50 per cent H₂SO₄. To the 3 cc. portion from Flask II the same amount of H₂SO₄ was added to keep the volume the same. After filtering each through a dry filter paper, 1 cc. portions of each of the clear filtrates were used for the indirect vanillin-HCl reaction. The colorimetric readings given in Table VI were obtained.

To test whether the hydrolysis had gone as far as it would, 2 cc. portions of U.S.P. pancreatin solution were added to Flasks III and IV, and 2 cc. portions of water were added to Flasks I and

Similar experiments upon another sample of casein and upon zein and autoclaved gelatin, gave 1.26, 0.19, and 0.12 per cent tryptophane, respectively. It should be stated, in order to make the colorimetric estimation more reliable, known amounts of tryptophane were added to the zein and gelatin estimations,

and the increased color over the amount added calculated as originating from the proteins.

The above figures are especially suggestive from a theoretical standpoint. If the casein molecule contains 1 molecule of tryptophane and the molecular weight of casein is of the order of 16,000, as based upon the sulfur and phosphorus content, then the tryptophane content should be 1.28 per cent. The values we find are of that order, whereas others have reported in the neighborhood of 1.5 per cent. Zein, on the basis of its sulfur content, assuming 3 atoms of sulfur per molecule, has a molecular weight of (15,983)_z. The 0.19 per cent of tryptophane found by us calls for a minimum molecular weight of 106,000. This is very unlikely, so that we are inclined to believe that the samples of zein examined by us, although usually considered pure, are nevertheless contaminated by other protein material higher in tryptophane content. If the impurity present in zein is a protein of the usual order of molecular weight and if it contains 1 molecule of tryptophane per molecule of protein, then we could infer as high as 14.8 per cent impurity in the zein. The same type of reasoning holds with respect to values we found on gelatin.

V.

Comparative Studies with the Phenol and the Vanillin-HCl Reagents.

At just about this stage of the work Folin and Looney (8) published their phenol method. They used the Ba(OH)₂ hydrolysis in their work and do not consider the possibility of tryptophane decomposition by such treatment. They also state that the acid treatment of tryptophane does not alter the tryptophane content as determined by the phenol reagent, thus claiming that all of the tryptophane decomposition products react identically as tryptophane does with the phenol reagent.

Accordingly, parallel experiments were conducted with the vanillin and phenol reagents and some interesting confirmatory results were obtained. Duplicates were carried out with the Folin-Looney procedure together with some modifications thereof.

300 cc. long necked Kjeldahl flasks were labelled I, II, III, IV, and V, respectively, and set up as in Table VIII.

Then 30 cc. of a 20 per cent (volume) H_2SO_4 solution were added to each of the five flasks and each was heated in a boiling water bath for 30 minutes (Folin-Looney's directions 30 to 60 minutes). The contents from Flasks I, II, and IV were filtered while warm into 100 cc. volumetric flasks. The BaSO_4 precipitates were washed with warm distilled water until the washings gave no test with the vanillin-HCl reagent. The flasks

TABLE VIII.

Flask No.	0.5 per cent tryptophane solution.	Casein 40 mesh.	$\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ per cent.	H_2O	Remarks.
	cc.	gm.	gm.	cc.	
I	3	0	3.5	25	Set aside at room temperature 40 hrs.
II	3	0	3.5	25	Heated on electric plate 40 hrs.
III	3	0	3.5	25	" " " " 40 "
IV	0	1	3.5	28	" " " " 40 "
V	0	1	3.5	28	" " " " 40 "

TABLE IX.

Standard.	I	II	III	IV	V
Vanillin-HCl reaction.					
15	Accurate comparison was impossible; all tubes, I to V, inclusive, showed an increasing amount of pink.				
Phenol reaction.					
15	15 15.1	16 16.2 16.1	15 15	15.6 15.6	14.5 14.5

were then cooled and made up to volume. III and V were treated as per Folin and Looney's directions; that is, the contents of each Kjeldahl flask were carefully transferred directly into 100 cc. volumetric flasks and rinsed 5 or 6 times with distilled water until a few drops of the rinsed water gave no test with the vanillin-HCl reagent. These flasks were cooled and made up to volume, then filtered through a dry quantitative filter into a dry flask.

It is desired to point out that the difference between the two procedures is that in the former (I, II, and IV) the BaSO_4 precipitate is removed before making up to volume, while in the latter, *i.e.* III and V, the bulky BaSO_4 precipitate is left in the flask and made up to volume. This latter procedure, as is seen below, introduces quite a large error.

A standard was prepared by using 3 cc. of the 0.5 per cent tryptophane solution and diluting to 100 cc. The mercury precipitation was carried out in duplicate, as usual, on the filtrates in Table VIII. One set was for the vanillin-HCl reagent, and the other for the phenol reagent. The mercury precipitation was carried out in the usual manner without any addition of the 50 per cent H_2SO_4 as the pH of the filtrate is supposed to be just right for the separation of tyrosine, etc. The colorimetric readings given in Table IX were obtained.

It is obvious that some substance other than tryptophane was present in all tubes, since a pink tint was prevalent and the color comparison by the vanillin-HCl reagent with the standard was impossible. On the other hand, with the phenol reagent all tubes showed the identical color, but not the same intensity of the standard, thus showing that some of the tryptophane decomposition products react similarly with the phenol reagent as with tryptophane itself, but also that some of the tryptophane is lost. Tubes II and III, as pointed out above, are identical in every respect except in the manner of making up to volume. This is also true of Tubes IV and V. As the colorimetric readings show, the difference in procedure introduces an error of about 7 per cent. This accounts for Folin and Looney's higher results on casein as well as for their failure to detect the loss of tryptophane in $\text{Ba}(\text{OH})_2$ hydrolysis.

We next took portions of the original hydrolysate from Flasks II, III, IV, and V and shook these well with the toluene. The toluene-shaken aqueous portions were labeled II_t, III_t, IV_t, and V_t, respectively, and the mercury precipitations thereon as well as on I, II, III, IV, and V were carried out similarly, but in a higher acid concentration. Thus, 1 cc. of 50 per cent H_2SO_4 was added to each tube and 3 cc. of the HgSO_4 reagent were added instead of 2 cc. as used by Folin and Looney. All the tubes were stoppered with rubber stoppers, shaken, and allowed

to stand for 2 hours, then centrifuged, decanted, washed with 5 per cent H_2SO_4 , centrifuged, decanted, and drained for $\frac{1}{2}$ minute as usual. The colorimetric readings of Table X were obtained.

Comparing the toluene-treated portions, II_t and III_t, with the toluene-untreated portions II and III, in both methods it is apparent that a trace of indole or skatole is precipitated by HgSO_4 even at that high acid concentration. As the readings indicate, the toluene-treated portions read higher than the toluene-untreated portions with both methods, and, in the case of the vanillin-HCl reaction, the latter shows a slight pink tint. Tube I is interesting in that $\text{Ba}(\text{OH})_2$ treatment in the cold and then acid treatment for 30 minutes in boiling water also causes some decomposition, but not nearly as much as in Tubes II and III.

TABLE X.

Standard.	III				III _t	IV	
Vanillin reaction.							
15	16	18.2	18.7	17	17.4	Reddish; cannot compare.	
	15.9	18	18.6	17	17.3		
	Slightly pink.		18.6	Slightly pink.			
Phenol reaction.							
15	16	18.6	19	17.0	17.8	17.8	17.0
	16	18.7	19	17.0	17.5	17.8	17.1
		18.7					

The impossibility of applying the vanillin reaction to IV and V in either case, whether the mercury precipitation has been conducted in lower or higher acidity and whether shaken with toluene or not, shows that some decomposition product other than indole or skatole is formed from casein by alkaline hydrolysis and that this is precipitated by mercury. It interferes with the vanillin reaction and gives the same color as tryptophane with the phenol reagent. This accounts for the higher results obtained for casein by the latter reagent. When the phenol reaction is carried out as above, *i.e.* mercury precipitation in higher acidity, but upon casein hydrolyzed by U.S.P. pancreatin, the results are the same as with the vanillin reagent.

It appears from these studies that the interfering products formed by alkali treatment of a protein (casein, gelatin, and zein) and alkali treatment of tryptophane itself are not the same, since in the latter we can remove them by shaking with toluene, but not in the former. Our results lead us to conclude that the reaction we employ is more specific for tryptophane and for unmodified tryptophane than reactions previously employed. By means of this more specific reaction we have been able to demonstrate very definitely the destruction of tryptophane in alkaline hydrolysis, and with the aid of our toluene separation we have given evidence that the nature of this decomposition in the case of pure tryptophane is in the main probably indole and skatole formation. However, in the case of the hydrolysis of a protein by boiling $\text{Ba}(\text{OH})_2$ we have formed not only indole and skatole but in addition, other decomposition products of tryptophane or other primary or secondary decomposition products, possibly not derived from tryptophane. Some of these products do not appear to give the same intensity of color as tryptophane with the phenol reagent of Folin and Looney.

The sensitivity of the vanillin-HCl reaction as compared with the phenol reaction on pure tryptophane with or without the mercury added is as follows: 1 mg. of tryptophane to 100 cc. with the phenol reagent gives a color intense enough for colorimetric comparison. 0.25 mg. of tryptophane to 50 cc. of vanillin-HCl gives a color intense enough for colorimetric comparison, hence sensitivity is 2:1. When compared similarly with *p*-dimethyl aminobenzaldehyde and pure tryptophane the relation is 3:2 in favor of the vanillin-HCl reagent.

VI.

Further Evidence as to the Presence of Tryptophane in Zein and Gelatin.

It is not surprising that with this more sensitive method we were able to detect tryptophane in proteins heretofore reported as free from this amino acid. However, it was felt that a confirmation by some other method or that the actual isolation of tryptophane would be very desirable. Thus 100 gm. of autoclaved gelatin and 50 gm. of zein, respectively, were subjected

to pancreatic hydrolysis in the usual manner, together with a control of the same amount of pancreatin. The hydrolysates were filtered, neutralized, and concentrated under vacuum to one-fourth the original volume. On cooling, tyrosine crystals came down in the zein hydrolysate. They were filtered off. The two concentrated hydrolysates were made up to volume, made acid (5 per cent by volume) with H_2SO_4 , and the mercury precipitation was carried out.

Although the mercury precipitate from the gelatin was about twice and that from zein about four times that of their respective controls of pancreatin alone, nevertheless it was impractical to work with a control which contained tryptophane to the extent of about 0.5 per cent as U.S.P. pancreatin does. Furthermore, it was impossible to wash the mercury precipitate free from tyrosine, even though the precipitation was carried out at as high an acidity as 8 per cent of H_2SO_4 . Folín and Looney (8) in their paper on the separation of tyrosine from tryptophane report that tyrosine will not be precipitated if the acidity is kept above 4 per cent of H_2SO_4 . This is true if the concentration of tyrosine is low, but from the principle of solubility-product it is clear that even if the mercury-tyrosine compound is more soluble than the mercury-tryptophane compound, nevertheless the precipitation of one in the presence of the other depends upon their relative concentrations of the solution in question. Tyrosine will come down if its concentration exceeds that required by the equilibrium ratio. Because of the tyrosine factor and the relatively large amount of tryptophane in the pancreatin this experiment was concluded as follows: The respective mercury precipitates were decomposed by H_2S in the usual manner and the indirect vanillin-HCl reaction was applied to equal aliquot portions of each.

Colorimetric readings:

Control set at.....	20
Zein.....	9.2
Gelatin.....	Reddish tint, could not compare.

This shows that more than two times as much tryptophane precipitated in the case of zein as in the control. Obviously, no definite comparison could be made with gelatin.

We next resorted to $\text{Ba}(\text{OH})_2$ hydrolysis, hoping that although some tryptophane would be decomposed, sufficient would remain to show its existence, since the control would be entirely negative in this case. Thus 100 gm. of gelatin and 50 gm. of zein were hydrolyzed with $\text{Ba}(\text{OH})_2$ according to Homer. The Ba was removed, then made up to proper pH, and the mercury precipitation carried out as usual. The next day the yellow precipitate was collected into 100 cc. centrifuge tubes, treated with 5 per cent H_2SO_4 , centrifuged, and decanted. This was repeated many times, but, nevertheless, the complete removal of the tyrosine was not accomplished. A portion of each precipitate, *i.e.* from the gelatin hydrolysate and from the zein hydrolysate, was tested with the glyoxylic acid ring test. A distinctly positive test was obtained with gelatin and a more positive one with zein. The precipitates were next suspended in 30 cc. of water and decomposed by H_2S ; the latter was removed by slightly warming and passing through a stream of CO_2 . The glyoxylic acid test was repeated and found positive as above. The bromine water test for free tryptophane and amyl alcohol extraction were next tried and found very positive for zein, but negative in the gelatin hydrolysate.

VII.

*A Study of the Effect of Synthetic Mixtures of Amino Acids
on the Quantitative Recovery of Tryptophane Directly
and When Incubated with U.S.P. Pancreatin
Solution.*

In view of the inability of obtaining a tryptophane-free protein to which to add a known amount of tryptophane in order to test the accuracy of the method on hydrolyzed protein, we next prepared mixtures of the following pure amino acids comparable to the concentrations reported to be in casein.

Three Erlenmeyer flasks, labelled 1, 2, and 3, respectively, were prepared as indicated in Table XI. The mercury precipitation and the vanillin-HCl reaction thereon were carried out upon 2 cc. portions of the mixtures. The first colorimetric readings given in the table show that the presence of the other

amino acid does not interfere with the quantitative estimation of tryptophane.

No.		Amino acid.
		<i>per cent</i>
1	Alanine.....	1.5
2	Leucine.....	9.4
3	Phenylalanine.....	3.2
4	Asparagine.....	1.4 (in place of aspartic acid).
5	Cysteine HCl.....	0.07
6	Cystine.....	0.10
7	Tyrosine.....	4.5
8	Ammonia.....	1.6
9	The diamino acid fraction was prepared in the usual manner from the acid hydrolysis of casein.	
	Arginine	13.38
	Histidine	
	Lysine	
10	Glycosamine-HCl was also added.....	10.0
11	Tryptophane.....	1.50

To each remaining portion add 0.2 gm. of Na_2CO_3 and 2 cc. of the filtrate of a 4 per cent U.S.P. pancreatin solution (throughout this work the pancreatic solution was filtered to insure uniform additions), then add toluene, and incubate at 35–37°C. Each day for the next 4 successive days equal portions of pancreatic solution were added to each flask. Then 5 cc. portions of each were made acid with 2 drops of 50 per cent H_2SO_4 , filtered through a dry filter paper, and 2 cc. portions of the filtrate then again used for the indirect vanillin-HCl reaction. The colorimetric readings "after incubation" in Table XI show the marked loss of tryptophane when the incubation is carried out in the presence of pancreatin and the other amino acids.

Certain observations led us to suspect the diamino acids and glucosamine to be involved in this loss. Accordingly, the following experiments were carried out with the results as given in Table XII. The concentrations of amino acids used were again of the same order as in the previous experiments.

A study of the results of Table XII shows that in the tryptic

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digestion of a protein we are likely to have a destruction or loss of tryptophane due to the interaction of the diamino acid fraction and glucosamine-HCl with tryptophane during this enzyme action. Flask 3, where the tryptophane was added after in-

TABLE XI.

A Study of the Effect of a Synthetic Mixture of Amino Acids on the Quantitative Recovery of Tryptophane Directly and When Incubated with U.S.P. Pancreatin.

Flask No.....	1	2	3
0.5 per cent tryptophane solution, cc.....	4	4	0
Amino acid mixture, cc.....	0	56	56
Water, cc.....	56	0	4
Colorimetric readings before incubation.....	15	{ 14.8 14.9	0
“ “ after “	15	{ 19.1 19.0	Trace.
Tryptophane lost, per cent.....	0	22	

TABLE XII.

Flask No.....	1	2	3	4	5	6
0.5 per cent tryptophane solution, cc...	4	4	4*	4	4	4
Amino acid solution complete.....	0	56	56	0	0	0
“ “ “ without diamino acids.....	0	0	0	43.6	0	0
Amino acid solution without glucosamine.....	0	0	0	0	46	0
Simple amino acids, no diamino acids, no glucosamine.....	0	0	0	0	0	33.6
Water.....	56	0	0	12.4	10	22.4
Na ₂ CO ₃ , U.S.P. pancreatin solution, per cent.....	The same amount added to each.					
Colorimetric readings.....	15	18.6	15	16.6	16.4	14.9
Loss of tryptophane, per cent.....	0	20	0	10	10	0

* In this case the tryptophane was added after the incubation.

cubation, shows no loss. Flask 2 shows the greatest loss of tryptophane, and it is interesting to note that the sum of the loss of tryptophane in Flasks 4 and 5, in the former due to the glucosamine-HCl and in the latter due to the diamino acid frac-

tion, is about the same as in Flask 2 which contains all of the amino acids.

To further study this remarkable action the following experiment was devised (Table XIII).

The last experiment was repeated, using glucosamine-HCl in place of the diamino acid fraction, and the results were the same.

The facts are that a loss of tryptophane is incurred if tryptophane is incubated together with U.S.P. pancreatin in the presence of either the diamino acid fraction or the glucosamine-HCl, or both. No loss is observed if incubated with pancreatin by

TABLE XIII.

Flask No.....	1	2	3	4
0.5 per cent tryptophane solution, cc.....	2	2	2*	0
Diamino acid solution, cc.....	5	5	5	0
Na ₂ CO ₃ solution, cc.....	20	20	20	0
U.S.P. pancreatin solution, cc.....	0	10†	10†	10†

The flasks were well shaken, preserved with toluene, and incubated at 35°C. as usual. At the end of the incubation period, 1 and 4 were combined and thoroughly mixed.

Colorimetric readings.....	20	21.6 21.7	19.9 20.0	
Tryptophane lost, per cent.....	0	8	0	

* Here the tryptophane was added after the incubation.

† Added in 2 cc. amounts on 5 successive days.

itself, or with pancreatin and the simple amino acids, or with the diamino acid fraction and glucosamine-HCl and no pancreatin. What the loss of tryptophane is due to and how the diamino acid fraction or glucosamine-HCl when incubated together with U.S.P. pancreatin changes or removes tryptophane we are not at present ready to say, but the subject is being investigated.

VIII.

CONCLUSIONS.

1. The vanillin reaction is specific for tryptophane, but certain tryptophane decomposition products give different tints.

2. Toluene has been found to separate quantitatively indole and skatole from tryptophane.

3. The vanillin reaction is more sensitive with tryptophane itself than any method previously cited.

4. By the vanillin reaction it was possible to detect tryptophane in zein and gelatin which usually have been considered as tryptophane-free.

5. Homer's $\text{Ba}(\text{OH})_2$ treatment of tryptophane has been shown by the vanillin reaction to introduce a loss of about 20 per cent; by the direct application of the phenol reagent without toluene extraction a loss of 7 per cent is introduced.

6. Different decomposition products appear in the $\text{Ba}(\text{OH})_2$ hydrolysis of proteins than in the $\text{Ba}(\text{OH})_2$ treatment of pure tryptophane; these products are carried down by the mercury precipitation of tryptophane.

7. Folin's method for separating tyrosine from tryptophane by mercury precipitation in sulfuric acid concentration above 3.5 per cent was found to hold only when the concentration of tyrosine is low, otherwise tyrosine also comes down.

8. Tryptophane, when incubated with the diamino acid fraction and U.S.P. pancreatin or glucosamine-HCl and U.S.P. pancreatin, has not been recovered quantitatively. The other simple amino acids do not prevent recovery of tryptophane.

9. Tryptophane has not been recovered quantitatively from proteins when hydrolyzed by acids, $\text{Ba}(\text{OH})_2$, or U.S.P. pancreatin.

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INORGANIC ALTERATIONS OF THE LYMPH IN CANINE ANAPHYLACTIC SHOCK.

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Anaphylactic shock in the dog, with its marked involvement of the splanchnic area, offers favorable opportunity for certain types of studies. The protracted period of shock permits alterations in the composition of the tissues and tissue fluids to take place, which are of interest not only in the study of anaphylactic shock, but also of shock phenomena of other origin, such as peptone or histamine shock, or that following injection of bacteria.

We have previously studied some of the alterations that take place in the thoracic lymph of dogs in anaphylactic shock (1). We have shown that immediately after injection of the antigen, alterations of the splanchnic endothelium take place whereby it becomes more permeable. This is followed by a reaction of the parenchymal cells in contact with the antigen which has passed through the normal endothelial barrier. The parenchymal cells chiefly concerned seem to be those of the liver.

While the endothelium in general is primarily involved in the reaction of anaphylactic shock, the endothelium of the liver seems to be particularly susceptible. In the dog, liver phenomena, such as coagulation changes, enzyme mobilization, and bilirubinemia, mark the picture as evidence of the striking change in the permeability of the hepatic endothelium. This seems to be associated with the peculiar structure of the liver endothelium, as illustrated and described by Zimmermann (2). In contrast to the ordinary endothelium of the capillaries, the liver endothelium forms a sieve, the individual cells containing small films with intervening ridges, films that are probably but a few molecular diameters in thickness. Such a system of ultrafilters may readily account for the rapidity with which even the formed elements

of the blood may pass from the capillaries into the lymph spaces when the endothelium is injured.

The rôle of the neurovascular apparatus in shock, particularly in anaphylactic shock, has become more and more apparent with the advancement of the knowledge of the basic phenomena involved in anaphylaxis. While most French workers have emphasized the purely physical changes that occur in serum in shock, others have endeavored to determine the cellular changes. Thus, Philippon, Mendeleeff, and Platounoff (3) have been able to determine alterations in the electrical resistance of the liver of guinea pigs in shock, alterations that take place very promptly, and even when the anaphylaxis was not very apparent. Manwaring and his associates (4) have clearly demonstrated the primary participation of the endothelium and the reaction of the liver and have postulated that the intoxication of the dog is due to the explosive formation of toxic substances in the liver cells. Coincidentally, numerous biologists have made apparent the relation of ionic changes associated with cellular stimulation, fatigue, and death. In this country Osterhout and Lillie, in Europe Zondek, Straub, Wiekowski, Billinghamer, and Embden, to mention but a few, have developed this field, and they have shown how the autonomic nervous system makes use of this primitive mechanism to correlate tissue activity. Zondek (5) and Dresel (6) show a correlated activity of the nervous system with the endocrine gland activity, and De Waele (7) has shown that anything that diminishes the relative vagus tone increases shock, while anything that increases the vagus tone diminishes shock. Dresel deals particularly with the changes of calcium and potassium concentrations in vagotonia and sympathicotonia. In vagotonia the tissues are more alkaline and contain less colloidal calcium and more potassium. The blood has little potassium, little ionized calcium, and more total calcium. In sympathicotonia all the changes are presumably reversed.

Alterations in the level of the mineral constituents of the lymph may reflect such changes in tissue activity, provided modification by large amounts of an ultrafiltrate from the blood capillaries does not overbalance the change brought about by the cellular activity. In anaphylactic shock, with its great increase in lymph flow, this latter factor is of great importance.

Methods.

Normal dogs weighing from 8 to 15 kilos were selected and given three injections of egg albumin intravenously on alternate days. The reinjection was made about 3 weeks after the final sensitizing dose had been given. All excitable and nervous animals were discarded. Thoracic incannulation was made under local anesthesia, and normal samples of lymph were collected. For the shock injection, fresh egg white was diluted to 40 per cent with physiological saline solution, filtered through cotton wool, and 20 cc. were used for intravenous injection.

Fibrin was determined by direct weighing after extraction with water, alcohol, and ether, and then dried. Chlorides were determined by the method of Folin (8). Conductivity was determined by the method of Kohlrausch (9). Results were expressed as resistance, since the same cell, with fixed electrodes, was used throughout the series of experiments. The remainder of the lymph was precipitated with trichloroacetic acid, and a Kjeldahl determination of nitrogen was made on the precipitate after washing. Phosphates were determined by the method of Bell and Doisy (10). Calcium was determined by precipitating as oxalate, washing, centrifuging, and titrating with potassium permanganate, the values in the protocols are expressed as calcium oxide. Magnesium was determined by precipitating as magnesium ammonium phosphate, washing, centrifuging, and determining the phosphate content colorimetrically, as suggested by Hammett and Adams (11). Sodium was determined by the method of Kramer and Tisdall (12). Potassium was determined by precipitation with sodium cobalti-nitrite, washing, and determining the nitrite content colorimetrically by the usual sulfanilic acid reduction method, as suggested by Briggs (13). Since lymph, like blood serum, contains extremely small amounts of potassium, and since our amount of material for analysis was very limited, the potassium values are expressed as the ratio of the potassium content of subsequent samples to the content of the first (normal) sample, and not in any absolute unit.

RESULTS.

In reviewing the results a number of factors must be considered. Any filtration of fluid to the lymph will modify its composition.

Mg per 100 c.c. lymph

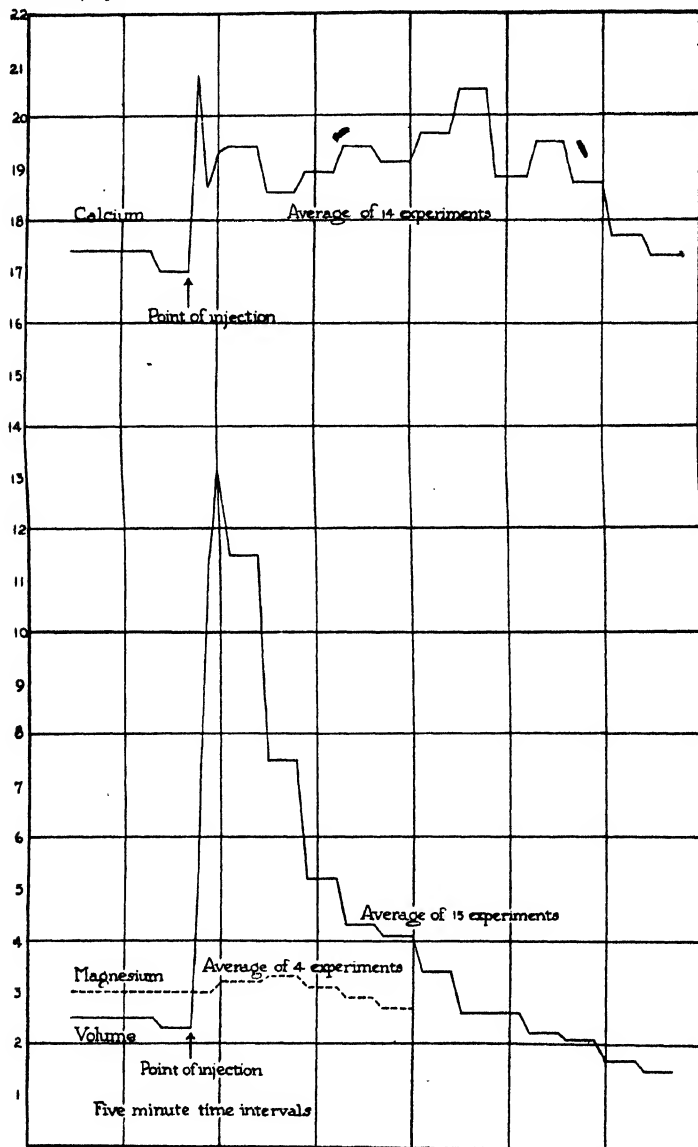


CHART 1. Volume of lymph flow; calcium and magnesium titers. Anaphylactic shock.

This applies particularly to anaphylaxis, where the lymph flow is increased tenfold. If the blood level of any salt is lower in the blood than in the lymph, then an increase of flow of blood fluid into the lymph will tend to lower the lymph level of that salt, regardless of the fact that the cells may be giving off more of that salt into the lymph than normal. The lymph exhibits considerable variations of its calcium and phosphate content under physiological conditions. Such variations occur even when the animal appears quiet, but are, no doubt, expressions of altered tissue activity under autonomic control. The other ions seem somewhat more stable in their concentration.

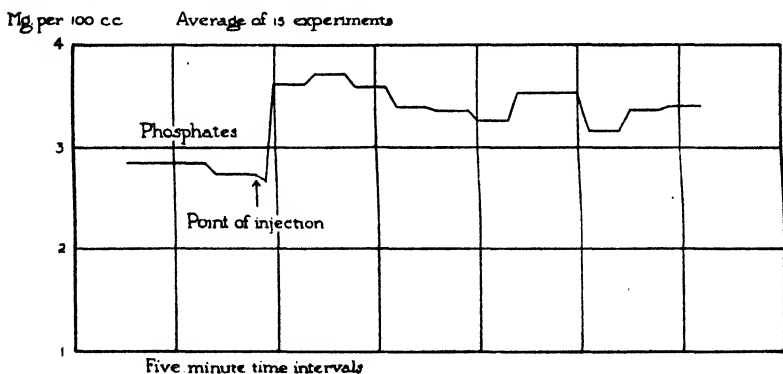


CHART 2. Phosphate titer of the lymph. Anaphylactic shock. Average of fifteen experiments.

Charts 1 to 3, which represent the averages of six experiments, each for the sodium, potassium, and magnesium content, and some sixteen experiments with calcium and phosphate determinations, are sufficiently clear-cut to bring out the chief alterations. Since these represent an average between severe and milder forms of shock they are not so striking as some of the individual changes shown in the appended protocols.

In the experiments which we have made, definite chemical evidence is brought to support the assumption that in anaphylactic shock cellular changes in the direction of increased cellular permeability take place. Calcium and magnesium leave the cell, as is shown by the increased concentration in the lymph, while sodium and potassium enter into the cell or cell membrane,

as shown by the decrease of these elements in the lymph. These reactions are practically instantaneous, since they become apparent usually in the lymph sample collected during the first 5 minutes after the injection of the antigen.

We may assume that such phenomena can occur only through the activity of the autonomic nervous system, or as a direct effect of the antigen when it comes in contact with the cell surface. The ionic alterations we observe in lymph after pilocarpine

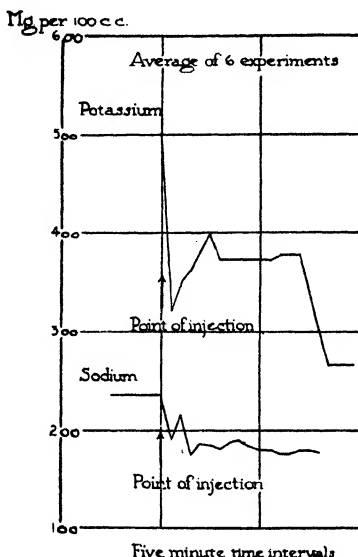


CHART 3. Sodium and potassium titers. Anaphylactic shock. The values for potassium are relative and have no relation to the marginal values indicated for sodium. Average of six experiments.

injection are similar to those in anaphylaxis; however, the absence of a disturbance of the coagulation mechanism, and the relatively moderate effect on the phosphate level indicates some differences in the mode of attack. It seems probable that the effect of moderate doses of pilocarpine are limited to the vascular endothelium and later the adventitial elements, while in the shock of anaphylaxis the antigen penetrates directly through the endothelium into contact with the parenchymal cells. These latter

respond with the more marked alterations such as acidosis, liberation of amino acids by proteolytic enzymes, increase in sugar, and disturbance of the coagulation mechanism.

The relation of anaphylactic shock to alterations in the relative tonicity of the sympathetic and parasympathetic nervous systems is illustrated in the experiments on the second series of dogs. When adrenalin was injected just before the toxigenic dose of the antigen an unusually severe shock followed and the calcium and magnesium of the lymph showed a most startling increase. When a previous injection of calcium chloride was made the animal died directly after the injection of the antigen.

Evidently an increase in the sympathetic tonus overbalances the parasympathetic system and makes the latter more labile.

The ionic alterations are those to be expected with cell stimulation, and make probable the conception of an intimate relationship of the autonomic nervous system in shock. The effects might, however, be achieved by the direct action of the antigen on the cells, without the intervention of the autonomic nervous system.

CONCLUSIONS.

The lymph in anaphylactic dogs reveals the following alterations: an immediate increase in lymph volume; an immediate increase in calcium; an increase in amino nitrogen; an immediate increase in magnesium; a decrease in the sodium level; a decrease in the potassium level; a progressive increase in phosphate, depending on the severity of the shock; and a constant level in chloride content.

Preliminary stimulation of the sympathetic system increases the effect of the shock.

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TABLE OF PROTOCOLS.

- Control series. A. Normal control. Protein, fibrin, calcium, phosphates, and amino N.
B. Normal control. Calcium and phosphates.
- First series. 1. Moderate anaphylactic shock. Calcium and phosphates.
2. Fatal shock. Calcium, phosphates, and amino N.
3. Moderate shock. Calcium, phosphates, and chlorides.
4. Moderate shock. Calcium, phosphates, and chlorides.
5. Moderate shock. Calcium, phosphates, and chlorides.
6. Moderate shock. Calcium, phosphates, and chlorides.
7. Moderate shock. Protein, fibrin, calcium, phosphates, and amino N.
8. Slight effect. Phosphates and amino N.
- Second series. 9. Slight shock. Protein, fibrin, calcium, phosphates, chlorides, and sodium.
10. Slight shock. Protein, fibrin, resistance, calcium, phosphates, chlorides, and potassium.
11. 1 cc. of 1:10,000 adrenalin intravenously before injection of antigen. Severe shock. Protein, fibrin, calcium, phosphates, magnesium, sodium, and potassium.
12. 10 cc. of normal calcium chloride, followed 5 minutes later by the antigen. Animal died 2 minutes after injection. No material for analysis. No protocol.
- Third series. 13. Severe shock. Protein, fibrin, resistance, phosphates, calcium, chlorides, magnesium, sodium, and potassium.
14. Fatal shock. Protein, fibrin, resistance, calcium, phosphates, chlorides, magnesium, sodium, and potassium.
15. Moderate shock. Protein, fibrin, resistance, calcium, phosphates, chlorides, magnesium, sodium, and potassium.
16. Severe shock. Protein, fibrin, resistance, calcium, phosphates, chlorides, magnesium, sodium, and potassium.

PROTOCOL A.

Weight 12 kilos. Normal control.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Calcium oxide.	Phosphates.	Amino N.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per cc.</i>	<i>mg. per cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per cc.</i>
1	15	12.6	4.2	45.38	1.58	15.3	3.85	0.239
2	15	9.5	3.2	42.72	1.52	14.2	3.85	0.212
Injected 20 cc. albumin solution.								
3	5	4.4	4.4					
4	5	3.0	3.0	64.49	1.89	15.2	2.70	0.595
5	5	4.0	4.0					
6	15	16.0	5.3	61.0	2.03		3.57	
7	15	13.2	4.4	48.74	1.74	15.4	3.45	0.141
8	15	16.8	5.6	55.31	1.31	14.2	3.23	0.163
9	15	14.6	4.9	47.5	1.30	15.3	3.13	0.226
10	15	9.5	3.2	45.86	1.26	15.4	3.23	0.229
11	15	4.6	1.5	43.6	1.3	14.6		0.261
12	15	6.6	2.2	44.24	1.24	13.6	3.23	0.230
13	15	5.4	1.8					
14	15	6.6	2.2	39.94	1.34	13.0	3.23	0.248
15	15	7.6	2.5					

PROTOCOL B.

Weight 14 kilos. Normal control. No symptoms.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	15	5.3	1.7	17.4	2.70
Intravenous injection of 20 cc. albumin solution.					
2	15	4.0	1.3	21.4	2.38
3	15	5.8	1.9	19.7	2.94
4	15	4.6	1.5	21.9	2.70
5	15	6.0	2.0	17.1	3.03
6	15	4.6	1.5		2.94
7	15	7.8	2.6	19.7	2.78
8	15	5.5	1.8	19.1	2.56
9	30	11.6	1.9	17.4	3.03
10	30	11.3	1.8	18.3	3.56
11	30	9.0	1.5	17.6	3.03

PROTOCOL 1.

Weight 12 kilos. Moderate anaphylactic shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	15	11.8	3.9	19.6	4.5
2	15	10.2	3.4	19.3	3.9
Injected 20 cc. albumin solution.					
3	5	5.2	5.2	33.6	4.0
4	5	12.0	12.0	17.6	3.7
5	5	16.3	16.2	15.4	3.8
6	15	44.0	14.7	17.2	3.9
7	15	20.0	6.7	16.6	3.8
8	15	15.0	5.0	17.3	3.6
9	15	12.0	4.0	22.2	3.8
10	15	10.0	3.3	23.0	3.4
11	15	9.6	3.2	24.7	3.8
12	15	5.8	1.9	34.5	3.8
13	15	6.0	2.0	20.1	3.4
14	15	4.4	1.5	22.0	3.4
15	15	5.6	1.7	19.1	4.0
16	15	5.0	1.7	22.8	4.7

PROTOCOL 2.

Weight 10 kilos. Fatal shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.	Amino N.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per cc.</i>
1	30	17.5	2.9	13.1	2.56	0.200
2	30	14.0	2.3	15.6	2.86	0.222
Injected 20 cc. albumin solution.						
3	5	6.5	6.5	23.5	2.50	0.291
4	5	13.4	13.4	20.0	2.80	0.380
5	5	9.4	9.4	17.8	3.00	0.388
6	15	19.5	6.5	17.4	4.20	0.595
7	15	24.4	8.1	20.7	5.71	0.621
8	15	17.9	6.9	18.2	5.42	
9	15	19.7	6.7	23.0	4.67	0.568
10	30	39.4	6.6	19.7	4.70	
11	30	39.2	6.6	17.1	5.48	0.466
12	30	35.3	5.9	18.5	5.26	
13	30	17.2	2.9	17.1	5.50	0.414
14	30	9.2	1.5	20.0	5.85	
15	30	8.5	1.4	23.0	6.23	0.336

Animal died.

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PROTOCOL 3.

Weight 12 kilos. Moderate shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.	Chlorides.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	30	8.0	1.7	18.5	2.08	810
Injected 20 cc. albumin solution.						
2	5	7.0	7.0	27.0	2.50	820
3	5	10.5	10.5		2.25	690
4	5	12.5	12.5	18.4	3.33	715
5	15	24.0	8.0	23.0	4.53	678
6	15	24.0	8.0	18.2	3.82	702
7	15	13.5	4.5	19.9	3.33	710
8	15	14.0	4.6	19.9	3.11	720
9	15	17.0	5.7	17.6	2.37	730
10	15	11.5	3.8	17.4	2.17	730
11	15	10.0	3.3	18.7	2.27	730
12	30	13.0	2.2	16.5	2.62	755
13	30	15.5	2.5	17.4	3.12	745
14	30	13.0	2.2	16.6	2.93	740
15	30	10.0	1.7	16.4	3.33	740
16	30	10.5	1.8	14.5	3.33	740

PROTOCOL 4.

Weight 12 kilos. Moderate shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.	Chlorides.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	30	10.5	1.8	17.3	2.50	575
Injected 20 cc. albumin solution.						
2	5	7.8	7.8	16.0	2.50	617
3	5	17.8	17.8	15.6	2.76	617
4	5	19.9	19.9	16.6	2.97	585
5	5	19.2	19.2	24.1	3.43	595
6	5	13.5	13.5		3.71	620
7	5	10.4	10.4		3.76	600
8	15	19.4	6.5	16.6	3.62	680
9	15	13.8	4.9	15.9	3.33	630
10	15	13.0	4.3	15.6	3.13	635
11	15	12.8	4.1	16.2	3.13	645
12	30	12.8	2.3	15.7	2.99	630
13	30	10.7	1.9	14.1	2.81	
14	30	8.7	1.5	12.6	2.78	
15	30	7.5	1.5	16.1	2.76	
16	30	6.5	1.2	14.7	2.53	
17	30	6.5	1.1	11.7	2.49	

PROTOCOL 5.

Weight 10 kilos. Moderate shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.	Chlorides.
	min.	cc.	cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	45	7.4	0.8	16.9	2.00	740
Injected 20 cc. albumin solution.						
2	5	4.8	4.8	21.6	2.02	
3	5	9.8	9.8	24.2	2.50	710
4	5	10.2	10.2	25.0	2.78	700
5	5	8.3	8.3	22.7	2.78	690
6	5	7.7	7.7	22.3	2.50	650
7	5	6.8	6.8	21.5	2.63	710
8	15	12.6	4.2	20.5	2.50	700
9	15	10.3	3.4	22.4	2.38	730
10	15	8.0	2.7	22.5	2.50	690
11	15	7.8	2.6	21.4	2.63	760
12	15	7.6	2.5		2.63	670
13	30	9.4	1.6	21.2	2.86	710
14	30	7.8	1.3	21.9	2.86	640
15	30	7.3	1.2		2.71	Lost.

PROTOCOL 6.

Weight 12 kilos. Moderate shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Calcium oxide.	Phosphates.	Chlorides.
	min.	cc.	cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	30	11.2	1.9	18.6	3.52	675
Injected 20 cc. albumin solution.						
2	5	13.0	13.0	19.7	3.49	670
3	5	15.1	15.1	19.5	3.84	675
4	5	13.5	13.5	27.2	4.17	670
5	5	14.2	14.2	20.8	5.00	665
6	5	17.0	17.0	20.3	5.68	670
7	5	15.0	15.0	19.3	5.63	650
8	5	13.0	13.0	20.3	5.21	620
9	5	14.0	14.0	19.8	4.34	645
10	5	8.7	8.7	19.5	3.96	670
11	15	20.2	6.1	22.1	3.81	650
12	15	13.4	4.5		Lost.	585
13	30	19.5	3.2	19.3	3.22	585
14	30	13.0	2.1	31.8	3.73	585
15	30	7.6	1.2	18.2	3.22	550

PROTOCOL 7.

Weight 13 kilos. Moderate shock. Some gastrointestinal symptoms.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Calcium oxids.	Phosphates.	Amino N.
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per cc.</i>	<i>mg. per cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	15	11.8	3.9	56.0	2.2	18.1	4.47	0.151
2	15	10.2	3.4	54.7	2.2	16.5	3.91	0.186
Injected 20 cc. albumin solution.								
3	5	5.2	5.2	54.0	0.7	14.9	4.03	0.277
4	5	12.0	12.0	63.0	0	19.6	3.70	0.158
5	5	16.2	16.2	59.5	0	17.8	3.83	
6	15	44.0	14.7	67.1	0	16.5	3.91	0.181
7	15	20.0	6.7	64.4	0.09	16.1	3.85	0.186
8	15	15.0	5.0	65.5	1.6	16.8	3.62	0.201
9	15	12.0	4.0	64.6	2.6	17.2	3.79	0.233
10	15	10.0	3.3	62.6	2.5	17.2	3.38	0.233
11	15	9.16	3.2	58.8	2.1	19.6	3.47	0.275
12	15	5.8	1.9		2.5	18.6	3.76	0.383
13	15	6.5	2.2	70.0	4.1	20.9	3.38	0.199
14	15	5.5	1.8					
15	15	4.4	1.5	55.7	3.8	17.2	3.57	0.214
16	15	4.4	1.5					
17	15	6.2	2.1	55.5	3.7	19.7	4.05	0.196
18	15	5.0	1.7					
19	15	6.3	2.1	55.2	5.4	20.3	4.72	0.214
20	15	4.4	1.5					

PROTOCOL 8.

Weight 10 kilos. Slight shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Phosphates.	Amino
	<i>min.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 10</i>
1	15	10.0	3.3	2.58	0.20
2	15	12.0	4.0	2.29	0.21
Injected 20 cc. albumin solution.					
3	5	4.6	4.6		0.33
4	5	7.5	7.5	2.42	0.12
5	5	6.75	6.75		0.10
6	10	11.5	5.7	2.72	0.20
7	15	25.0	8.3	3.25	0.20
8	15	25.0	8.3	3.29	0.21
9	15	19.5	6.5	3.17	0.24
10	15	22.0	7.4	2.88	0.20
11	5	17.5	5.7	2.65	0.20
12	15	13.5	4.5	2.65	
13	15	13.5	4.5	2.65	0.21

PROTOCOL 9.

Weight 7 kilos. Slight shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Calcium oxide.	Phosphates.	Chlorides.	Sodium.
	min.	cc.	cc.	mg. per cc.	mg. per cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	15	7.6	2.5	63.8	1.62	17.4	3.58	690	257
2	15	6.0	2.0						
Injected 20 cc. albumin solution.									
3	5	2.5	2.5	83.0	3.23	20.0	2.86	692	268
4	5	10.0	10.0						
5	5	4.6	4.6	79.9	2.93	19.9	3.32	697	203
6	15	10.0	3.3						
7	15	9.8	3.3	74.6	2.64	18.6	3.13	701	235
8	15	8.2	2.7						
9	15	8.1	2.7	71.8	2.50	19.4	3.13	700	173
10	15	6.7	2.2						
11	15	6.5	2.2	62.7	1.79	20.0	3.32	700	92
12	15	9.0	1.5						
13	15	1.2	2.0	64.2	1.94	19.2	3.58	702	182
14	15	7.6	1.2						
15	15	9.3	2.6						

PROTOCOL 10.

Weight 12 kilos. Slight shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Resistance.	Calcium oxide.	Phosphates.	Chlorides.	Potassium in terms of 100.
	min.	cc.	cc.	mg. per cc.	mg. per cc.		mg. per cc.	mg. per 100 cc.	mg. per 100 cc.	
1	15	4.3	1.4	49.7	1.13	45.5	17.1	2.33	707	1.00
2	15	7.0	2.3							
3	15	4.5	1.5	68.6	2.99	44.6	16.7	1.99	686	54.2
4	5	2.0	2.0							
5	5	4.5	4.0	67.2	3.15	45.4	13.1	2.56	690	54.2
6	5	8.2	8.2							
7	15	24.0	8.0	67.2	3.24	45.4	18.6	2.58	690	66.0
8	15	17.0	5.7							
9	15	9.0	3.0	57.6	2.33	48.0	18.9	2.08	695	54.2
10	15	7.3	2.4							
11	15	6.6	2.2	53.8	1.80	43.4	19.5	1.70	710	53.8
12	15	7.3	2.4							
13	30	23.0	1.5	49.4	1.42	40.6	14.8	1.92	710	63.0
14	45									

Weight 14 kilos. Severe shock.

[illegible]

Weight 12 kilos. Severe shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Resistance.	Calcium oxide.	Phosphates.	Chlorides.	Magnesium.	Sodium.	Potassium (comparative values).
	min.	cc.	cc.	mg. per cc.	mg. per cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	15	12.0	4.0	28.85	0.55	39.8	12.5	2.05	720	3.0	235	0.556
2	15	6.2	2.0									
Injected 20 cc. albumin solution.												
3	5	8.0	8.0	39.2	0.3	42.8	14.05	2.08	712		171	0.357
4	5	12.5	12.5									
5	5	21.0	21.0	47.53	0.33	41.3	16.81	3.11	682	3.74	153	0.213
6	5	22.0	22.0	49.38	1.28	41.6	17.58	3.07	670	3.97	135	0.25
7	5	18.0	18.0	52.0	2.2	42.8	16.3	2.99	680	3.97	144	0.416
8	5	17.0	17.0	49.53	1.83	42.4	16.3	2.66	678	3.97	108	0.358
9	5	15.5	15.5	48.86	1.86	42.4	16.3	3.13	667	4.2	117	0.313
10	5	14.0	14.0	48.4	2.2	45.3	16.15	3.11	668	4.76	117	0.313
11	5	13.0	13.0	47.6	3.2	46.5	18.1	3.07	670	4.36	117	0.358
12	15	23.5	8.0	41.45	1.45	47.4	17.0	2.94	680	3.58	90	0.385
13	15	14.0	4.8	42.14	2.14	45.3	18.8	2.63	685	3.74	90	0.333
14	15	13.0	4.3	45.06	5.16	44.9	17.8	2.63	696	3.74	98	
15	15	10.8	3.6		2.96							
16	15	8.6	2.9	43.36	3.96	46.1	17.45	2.44	705	3.58		
17	15	7.5	2.5	39.5	5.46	43.8		2.39	705			
18	15	7.0	2.3		3.43							

PROTOCOL 14.

Weight 15 kilos. Fatal shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Resistance.	Calcium oxide.	Phosphates.	Chlorides.	Magnesium.	Sodium.	Potassium (relative values).
	min.	cc.	cc.	mg. per cc.	mg. per cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	15	12.0	4.0	40.0	1.75	47.6	19.1	2.38	765	4.00	180	0.24
2	15	12.8	4.3		1.08							
3	15	14.0	4.8		1.57							
Injected 20 cc. albumin solution.												
4	5	11.8	11.8	42.1	1.52	46.1	16.6	3.12	765	2.92	162	0.20
5	5	13.0	13.0	52.4	1.96	48.6	17.1	2.50	775	3.08	198	0.30
6	5	22.0	22.0	57.2	0.80	47.2	13.6	2.94	770	3.29	171	0.38
7	5	23.0	23.0	58.8		45.6	14.5	3.22	775	3.29	189	0.38
8	5	21.0	21.0	62.4		48.6	14.5	3.57	760	2.78	163	0.38
9	5	20.5	20.5			49.6	17.5	4.16	760	2.92	163	0.34
10	5	16.0	16.0	54.7		47.6	16.8	4.54	765	2.92	180	
11	5	12.0	12.0	46.6	0.1	46.0	15.8	4.75	765	3.27	189	
12	5	11.6	11.6									
13	15	20.0	6.66	50.4	0.2	47.2	17.7	5.55	760	2.92	189	0.26
14	15	15.0	5.0	32.0	0.8	47.6	15.6	5.88	755	3.29	189	
15	15	13.0	4.3	42.4	1.38	44.2	14.9	6.75	740		208	
16	15	11.6	3.8	43.0	1.48	45.0	17.8	7.14	785	3.29		0.40
17	15	7.0	2.3	43.1	1.54	45.0	17.9	8.32	780	3.29		0.40
18	15	4.0	1.3									

Animal died.

Weight 8 kilos. Moderate shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Resistance.	Calcium oxide.	Phosphates.	Chlorides.	Magnesium.	Sodium.	Potassium (relative values).
	min.	cc.	cc.	mg. per cc.	mg. per cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	15	9.0	3.0	32.7	0.8	40.0	15.8	2.85	610	2.09	171	0.71
2	15	4.2	1.3									
Injected 20 cc. albumin solution.												
3	5	9.2	9.2	41.8	0	40.4	18.3	2.77	640	1.80	180	0.52
4	5	9.4	9.4	48.7	0	41.0	19.5	2.70	660	2.38	189	0.59
5	5	8.0	8.0	45.0	0	40.0	17.9	3.57	670	2.38	162	0.67
6	5	9.6	9.6									
7	5	8.6	8.6	45.0	0	39.4	18.7	5.26	660	2.60	198	0.62
8	5	6.6	6.6									
9	5	4.6	4.6	42.5	0	40.4	18.7	5.88	670	2.61	198	0.55
10	5	4.2	4.2									
11	15	10.3	3.4	39.7	0.34	40.0	17.2	4.44	660	1.85	171	
12	15	11.3	3.7	38.6	0.52	39.0	19.3	3.57	660	1.85	153	0.71
13	15	11.5	3.8	37.4	1.77	38.4	15.7	3.03		1.72	144	0.91
14	15	9.8	3.3									
15	15	8.0	2.7	37.0	1.68	40.0	15.6	2.94	294	1.80	180	0.91
16	15	8.0	2.7									
Blood before				47.5			17.3	2.85	660	1.93		
" 5 min. after . . .				45.6		39.3	17.2	3.28	680	2.02		0.43
" 20 " " } . .				45.6			19.8	2.84	710	2.27		
" 1 hr. " }												

PROTOCOL 16.

Weight 15 kilos. Severe shock.

Sample No.	Time.	Volume.	Volume per 5 min.	Protein.	Fibrin.	Resistan e	Calcium oxide.	Phosphates.	Chlorides	Magnesium.	Sodium.	Potassium (rela- tive values).
	min.	cc.	cc.	mg. per cc.	mg. per cc.		mg. per 100 cc	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	15	6.3	2.1	49.2	1.0	42.6	19.6	2.63	675	3.94	225	0.143
2	15	7.0	2.3									
Injected 20 cc. albumin solution.												
3	5	10.0	10.0	55.1	0.1	45.4	22.0	2.50	695	4.18	162	0.100
4	5	13.0	13.0	58.7	0.5	45.3	19.3	2.38	680	3.52	163	0.094
5	5	15.0	15.0	68.4	0.8	49.5	18.3	2.85	675	3.5	171	0.156
6	5	12.2	12.2	65.1	1.3	47.2		3.45	690		207	0.200
7	5	16.0	16.0	54.8	2.3	47.7	17.6	3.10	685		180	
8	5	10.8	10.8	60.3	2.1	48.0	16.0	3.10	695	3.54	189	0.143
9	15	18.0	6.0	65.9	2.8	45.0	14.8	2.73	695	3.18	180	0.135
10	15	15.0	5.0	52.3	2.3	47.7	17.7	2.17	685		207	0.147
11	15	12.5	4.2	47.4	2.6	44.0	13.4	1.92	695	3.08	198	0.114

OBSERVATIONS ON BLOOD LACTIC ACID AFTER INSULIN.*

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INTRODUCTION.

The fate of the sugar which disappears from the blood under the influence of insulin is still very obscure. The interesting conclusion reached by Briggs, Koechig, Doisy, and Weber (1) that there is an increase in lactic acid parallel with the decrease in dextrose seemed worthy of further investigation. Although, in some experiments, these workers found a marked increase in lactic acid which was accompanied by a fall in dextrose, in certain cases the decrease in sugar was not coincident with a significant rise in blood lactic acid, and in a few instances a marked rise in lactic acid was accompanied by only a slight decrease in blood sugar. In these experiments, blood samples were secured from the femoral artery, before and several hours after the administration of insulin. When the second sample was obtained the animal was in the drowsy asthenic condition which may precede hyperirritability. Hepburn, Latchford, McCormick, and Macleod (2) have reported one experiment in which the blood sugar of an etherized dog was markedly lowered by insulin without any significant increase in lactic acid. The recent experiments of Ronzoni, Koechig, and Eaton (3) should be considered in connection with lactic acid studies on etherized dogs.

Since the blood sugar of normal dogs may be reduced very rapidly and to very low levels without the occurrence of marked asthenia or hyperirritability it appeared advisable to study blood lactic acid before the appearance of either of these complicating

* A summary of this paper was presented at the December, 1924, meeting of the American Physiological Society.

TABLE I.
Normal Dog.

Date.	Time inter- val		Blood sugar.	Lactic acid.	Difference in blood sugar.	Difference in lactic acid.	Remarks.
1924	hrs.	min.	per cent	mg. per 100cc.	mg.	mg.	
Sept. 16			0.088	38			Dog was fasted for 24 hrs. previ- ous to each experiment. Lac- tic acid is calculated on an average of at least three titra- tions, using Clausen's method.
" 18			0.092	38.5			
Oct. 7			0.086	26			
" 8			0.107	45.5			
Sept. 20			0.088	23.5			A volume of physiological saline solution equal to that of in- sulin in the succeeding experi- ments was injected.
	1	30	0.084	23.0	-4	-0.5	
		30	0.092	21.5	+4	-1.5	
Oct. 9			0.104	45.5	-6	-5	
		30	0.098	40.5			" "
" 10			0.098	39			" "
		30	0.094	38	-4	-1	" "
" 11			0.088	33			" "
		30	0.084	29	-4	-4	" "
" 13			0.098	43.5			" "
		30	0.088	35.5	-10	-8	" "
Nov. 11	2	30	0.084	17			A volume of inert insulin solution equal to that of the active ma- terial used in succeeding ex- periments was injected.
		30	0.079	15	-5	-2	
" 12	2	30	0.079	26			
		30	0.084	25	+5	-1	
" 13	2	30	0.085	23.5			" "
		30	0.079	22.5	-6	-1	" "
" 17	2	30	0.088	18.5			" "
		30	0.082	21	-6	+2.5	" "
" 18	2	30	0.088	21.5			" "
		30	0.079	21	-9	-0.5	" "
" 19	2	30	0.088	16.5			" "
		30	0.084	17.5	-4	+1	" "

TABLE II.
Normal Dog.

Date.	Time inter- val.		Blood sugar	Lactic acid	Difference in blood sugar.	Difference in lactic acid	Remarks.
	hrs.	min.	per cent	mg.	mg.	mg.	
1924							
Oct. 20		15	0.088 0.057	25 27.5	-31	+2.5	8 units of insulin injected.
" 21		15	0.088 0.051	18.5 19	-37	+0.5	10 " " " "
" 24		15	0.085 0.044	20.5 22	-41	+1.5	10 " " " "
" 25		15	0.092 0.040	20.5 21.5	-52	+1	10 " " " "
" 14		30	0.092 0.048	33 39	-44	+6	15 " " " "
" 15		30	0.092 0.044	43 49	-48	+6	15 " " " "
" 16		30	0.088 0.048	41.5 35.5	-40	-6	15 " " " "
Nov. 21		30	0.088 0.048	22 24.5	-40	+2.5	15 " " " "
" 28	2		0.088 0.054	21 23	-34	+2	10 " " " "
Dec. 8	1	30	0.088 0.054	16.5 25.5	-34	+9	30 " " " " Animal was drowsy and as- thenic after insulin.
" 9	1	30	0.088 0.063	15.5 21.5	-25	+6	15 units of insulin injected.
" 11	1 4		0.088 0.044 0.048	16 20.5 16	-44 -40	+4.5	80 " " " " Dog was very weak after the 4th hr.
Nov. 24	4		0.087 0.085	21 20	-2	-1	10 units of insulin injected.
Dec. 26	1 5	15	0.092 0.054 0.018	7 9.5 19.5	-38 -74	+2.5 +12.5	100 " " " " In 1½ hrs. the animal was bright, but at 5 hrs. was semicoma- tose.

TABLE III.
Diabetic Dog.

Date.	Time inter- val.		Blood sugar.	Lactic acid.	Difference in blood sugar.	Difference in lactic acid.	Remarks.
	hrs.	min.	per cent	mg.	mg.	mg.	
1924							
Dec. 5	4	30	0.221 0.048	23.3 29.0	-173	+5.7	Diabetic dog fasted 24 hrs. previous to experiments and injected with 80 units of insulin. Animal was very weak when the second blood was obtained.
" 7	1		0.174 0.072	19 22.5	-102	+3.5	100 units of insulin injected. Dog was very weak at the end of the 1st hr.
" 17	2	30	0.250 0.085	24 28	-165	+4	40 units of insulin injected.
" 22	2		0.180 0.136	26 33	-44	+7	10 " " " "

TABLE IV.

Date.	Difference in blood sugar.	Difference in lactic acid.	Difference in Hb.	Remarks.
1924	mg.	mg.	per cent	
Nov. 20	-34	+2	+16.6	10 units of insulin injected into a normal dog. Second sample taken in 2 hrs.
Dec. 8	-30	+9	+15.3	30 units of insulin injected into a normal dog. Second sample taken in 1½ hrs.
" 9	-25	+6	- 0.3	15 units of insulin injected into a normal dog. Second sample taken in 2 hrs.
" 10	-44	+4.5	+13	80 units of insulin injected into a normal dog. Second sample taken in 1 hr.
" 7	-102	+3.5	+17.6	100 units of insulin injected into a diabetic dog. Second sample taken in 1 hr.
1925				
Jan. 2	Lost.	+5	+19	50 units of insulin injected into a normal dog. Second sample taken in 4 hrs.

The normal hemoglobin value was assumed to be 100 per cent. The differences in hemoglobin as listed in the above table are the variations from the assumed normal.

factors. We have, therefore, estimated sugar and lactic acid of the blood before insulin and at varying intervals from 15 minutes to 5 hours after the administration. A marked fall in blood sugar was always noted and in many experiments the animal was free from asthenia or hyperirritability.

Methods.

Blood lactic acid was estimated by Clausen's method (4). Considerable study and numerous control experiments were necessary before satisfactory results were uniformly secured. Added lactic acid could then be recovered in practically the same amounts as noted by Clausen and by other investigators who have used this method (5). We have employed the permanganate procedure in all experiments. All estimations were made in triplicate and the lactic acid value is in each case the average of three determinations. We have further checked our technique and the method by studying blood lactic acid after severe insulin convulsions in rabbits, and after the injection of sodium carbonate in dogs (6). Marked increase in blood lactic acid was noted under both conditions. Blood sugar was estimated by the Shaffer-Hartmann method (7). The hemoglobin concentration was followed in some experiments. The carbon monoxide saturation procedure was used.

Specially trained normal dogs were used. The blood samples were secured from the saphenous vein by puncture through the skin and insulin was usually introduced by this route. This procedure was well tolerated by the animals and was unaccompanied by struggling or any signs of excitement. Dogs were depancreatized by the procedure described in the early reports on insulin. Blood samples were immediately delivered into distilled water, so glycolysis could not be responsible for the formation of any part of the lactic acid.

DISCUSSION.

The figures obtained for normal lactic acid content of dog's blood (8) are interesting in that they show the variation from day to day. Since only two normal dogs were used there is little data on the average normal value for dogs. In Table I a series of control experiments is listed. The results show that lactic

acid of the blood is not increased or significantly changed by saline or inert insulin solution. In the experiments in which active insulin was administered a slight rise in blood lactic acid was usually noted (Table II), but the increase is in most experiments slight and in no case would it account for more than a small percentage of the dextrose which disappears, if the blood dextrose could be considered the source of the acid. This discrepancy is particularly marked in the experiments on diabetic dogs (Table III). In several experiments on normal dogs in which the animal became prostrated and appeared very drowsy the lactic acid was found to have increased more than in the experiments where the subject remained bright throughout.

In a number of experiments the concentration of hemoglobin was studied. In many cases a definite increase in hemoglobin was noted, in both normal and diabetic dogs. Our positive results, which were not invariably obtained in the normal dog, are similar to those of Drabkin and Edwards (9). Haldane, Kay, and Smith (10) have reported that the administration of insulin to normal rabbits leads rapidly to an appreciable rise in blood volume. This is certainly not the case in dogs. Olmsted and Taylor (11) noticed a slight fall or no change in the hemoglobin value in rabbits after insulin. Very recently, Hamilton, Barbour, and Warner (12) have reported that insulin causes no significant change in the specific gravity or total solids in the blood of normal dogs and rabbits. In view of these discordant results the significance of the hemoglobin changes after insulin is not as yet clear (Table IV).

CONCLUSION.

The lactic acid of normal or diabetic dogs' blood, as determined by Clausen's method, does not significantly increase during insulin hypoglycemia when this condition is uncomplicated by extreme asthenia or marked hyperirritability.

Addendum.—In the introduction of this article we neglected to refer to a communication, by Tolstoi, Loebel, Levine, and Richardson (13). These investigators found no quantitative relationship between blood sugar and lactic acid production in diabetic patients.

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THE RELATIVE VALUE OF ULTRA-VIOLET LIGHT AND IRRADIATED AIR IN PREVENTING RICKETS IN CHICKENS.*

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(Received for publication, December 9, 1924.)

During the time experiments¹ were being conducted at the Kansas Experiment Station on the influence of ultra-violet light on leg weakness (rickets) in growing chicks, Hume and Smith (1923) reported² the results of an experiment from which they concluded that much of the benefit of the ultra-violet light in preventing rickets in rats is due to its action on the air which the animals breathe. This view that the irradiated air is of as much or more value in promoting normal bone development than the direct action of the ultra-violet light on the animal is in accord with the view of many poultrymen who believe that the fresh air the chickens receive out of doors is of more value in preventing leg weakness (rickets) than the direct sunlight. These conclusions are not in harmony with our experimental results. The experiment described in this paper was conducted to determine the importance of irradiated air in the prevention of rickets in growing chicks.

Before this experiment was completed Webster and Hill (1924) reported³ the results of an experiment from which they conclude that the beneficial action of ultra-violet light in preventing rickets in rats is due to its direct action on the animal's body and not to any chemical change produced in the air which the animal

* Contribution No. 108, Chemistry Department, Kansas State Agricultural College, Manhattan.

¹ *Kansas City Weekly Star*, 1923, xxxiv, 1. Hughes, J. S., *Science*, 1924, lix, 213.

² Hume, E. M., and Smith, H. H., *Biochem. J.*, 1923, xvii, 364.

³ Webster, T. A., and Hill, L., *Biochem. J.*, 1924, xviii, 340.

breathes. Our results, which are in accord with those of Webster and Hill, show that irradiated air has no value in preventing rickets in growing chicks.

For this experiment a group of day old White Leghorn chicks was obtained from the College Poultry Farm. They were all kept together in our nutrition room for 3 weeks, during which time they received a ration of 90 parts of basal feed plus 10 parts of clipped oat sprouts. The basal ration consisted of ground grain, 90 parts, and tankage, 10 parts. This mixture of the basal ration and oat sprouts was made fresh at each feeding, which was done twice daily, and was ground together so intimately that the chicks could not separate the oat sprouts and the grain. This ration is one on which chicks will develop rickets in from 5 to 10 weeks if they receive no ultra-violet light.

TABLE I.
Percentage Composition of Bones Calculated on Dry Basis.

	Lot I.	Lot II.	Lot III.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calcium.....	5.75	6.85	5.76
Phosphorus.....	2.86	3.36	2.96

At 3 weeks of age the chicks were leg banded and divided into three lots of twelve each. Lot I was kept as a control. Lot II was given a 10 minute treatment twice daily of ultra-violet light from a quartz mercury arc lamp. Lot III was given a treatment of irradiated air for a 10 minute period twice daily.

The apparatus in which the light and air treatment was given consisted of a box made in the shape of an inverted letter L (Fig. 1). The vertical compartment of the box is 4 feet high and the horizontal compartment is 2 feet long. The mercury arc lamp was placed over an opening in the top of the box so that its light was directed downward into the vertical section. All light was excluded from the horizontal compartment by means of a series of baffles, painted a dead black, which were placed between the vertical and horizontal compartments. These baffles were arranged in such a manner as to allow a current of air blown up through the vertical compartment to pass out through the horizontal compartment.

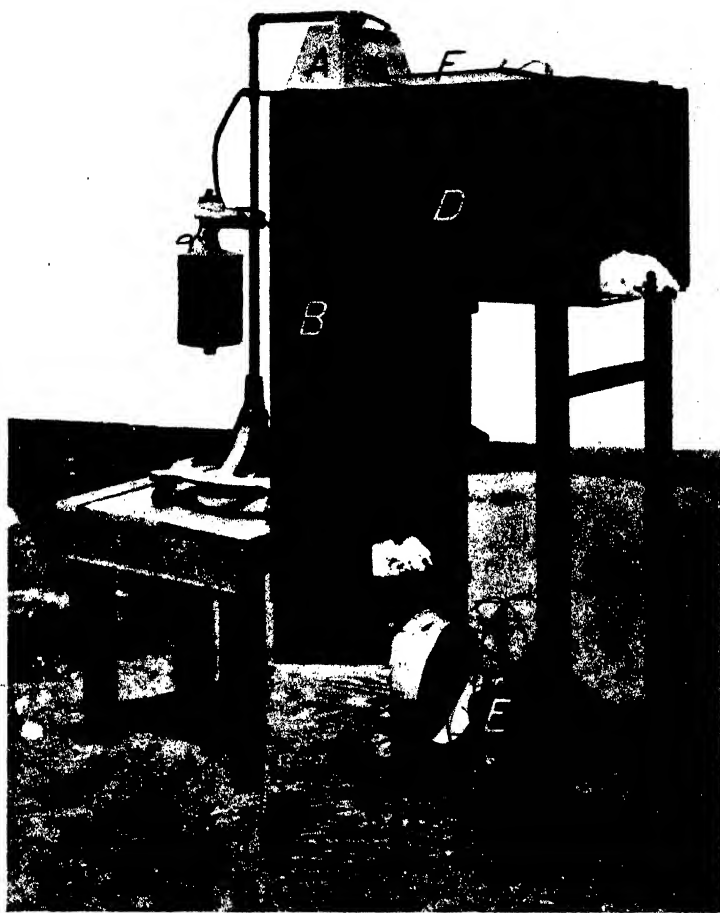


FIG. 1. Photograph of apparatus used in treating chickens with ultra-violet light and irradiated air. *A* is a lamp loaned by the Cooper-Hewitt Electric Company taking 3.5 amperes at 200 volts; *B*, vertical compartment in which chicks received direct light from Lamp *A*; *C*, door to compartment *B*; *D*, horizontal compartment in which chicks were surrounded by irradiated air blown up through *B* by electric fan *E*; *F*, door to compartment *D*.



Lot I.



Lot II.



Lot III.

FIG. 2. Photograph of the three lots of chicks used to determine the relative value of ultra-violet light in irradiated air in preventing rickets. Photograph taken at end of 8 weeks of the experiment. All lots received the basal ration of ground grain and tankage plus 10 per cent oat sprouts.

Lot I. Control lot. Received no ultra-violet light or irradiated air. All chicks show development of rickets. Note similarity to Lot III.

Lot II. Received direct ultra-violet light. The chicks were treated for 10 minute periods twice daily to direct exposure to ultra-violet light. Note size as compared with Lots I and III. Also note that all chicks are standing and that no signs of rickets are in evidence.

Lot III. Received irradiated air treatment for 10 minute periods twice daily. Note that irradiated air had no beneficial effects and that the chicks are in no better condition than Lot I. From this we conclude that irradiated air has no influence in preventing rickets (leg weakness) in growing chicks.

During the treatment the chicks in Lot II were placed on a screen at the bottom of the vertical compartment and the chicks of Lot III were placed in the horizontal compartment. In this way Lot II received the direct ultra-violet light and at the same time was continually surrounded by fresh non-irradiated air blown up through the vertical compartment by an electric fan. At the same time Lot III received no ultra-violet light, but was continually surrounded by a current of air which had been irradiated while passing up through the vertical compartment.

At the end of the 8 weeks of the experiment all the chicks in Lot I, the control lot, and those in Lot III, which received the irradiated air had developed rickets. None of those in Lot II, which received the ultra-violet light, developed this condition. Five chicks died in each of Lots I and III. None died in Lot II. We have conducted three different experiments on the value of ultra-violet light and irradiated air, and all have shown similar results.

The general condition of the chicks at the end of the 8 weeks is shown in Fig. 2. At the end of the experiment the chicks were killed and an analysis was made of the bones. The results are shown in Table I.

From these experiments we conclude that the beneficial action of ultra-violet light in preventing rickets in chickens is due to its direct action on the chickens and not to the changes which it produces in the air the chickens breathe.

DIETARY REQUIREMENTS FOR REPRODUCTION.

IV. SOLUBILITY OF THE REPRODUCTIVE DIETARY COMPLEX (VITAMIN E) IN VARIOUS ORGANIC SOLVENTS.*

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(From the Department of Agricultural Chemistry, Agricultural Experiment Station, University of Arkansas, Fayetteville.)

(Received for publication, January 19, 1925.)

In a recent communication evidence was presented¹ showing that ether extracts from wheat embryo, yellow corn, and hempseed, a hitherto unrecognized organic factor, designated as vitamin E, essential for reproduction. In this paper experimental data are submitted showing that vitamin E is soluble not only in ether, but also in benzene and acetone.

The results of the experiments are indicated in Charts I to VII and Figs. 1 to 3.

CHART I, LOT 467. This chart shows the growth of a second generation of males born of Female 1995, which had a skimmed milk powder ration containing 3 per cent of an ethereal extract of wheat embryo. Several second generation males and females have now grown up to sexual maturity on similar rations. Twelve females partaking of rations alike in every respect to that consumed by Lot 467, but having no vegetable oil present, did not even become pregnant.

FIG. 1. This figure shows two healthy second generation males of Lot 467, grown up to sexual maturity.

CHARTS II and III, LOT 503. These charts clearly demonstrate that acetone extracts from the germ of wheat an organic complex potent not only for fertility but also for successful lactation.

FIG. 2. This figure shows a healthy, vigorous litter of six young, 24 days old, reared normally throughout the lactation period and successfully

* An abstract of this paper was presented before the American Society of Biological Chemists at Washington, D. C., December 30, 1924, under the title of "Positive evidence for the existence of the reproductive dietary complex (vitamin E) soluble in benzene, ether, and acetone."

Research paper No. 18, Journal Series, University of Arkansas.

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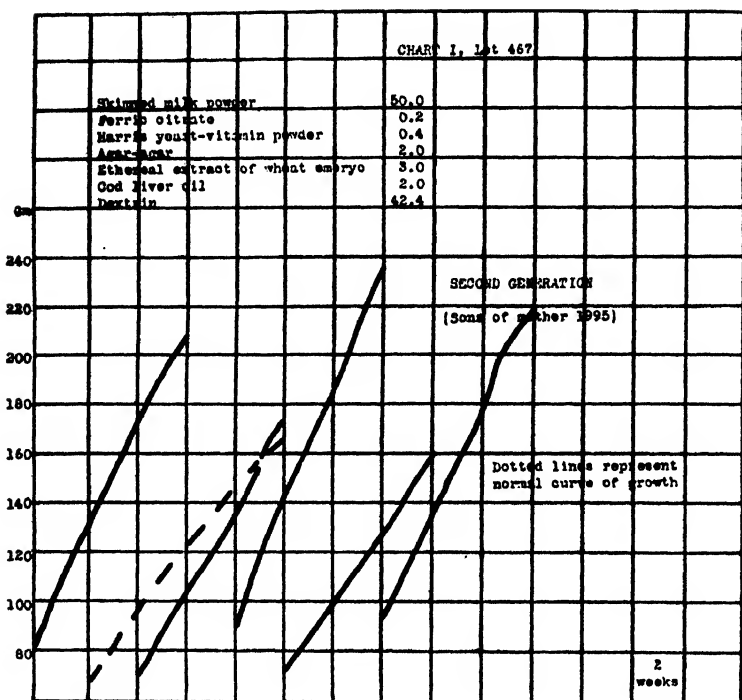


CHART I.

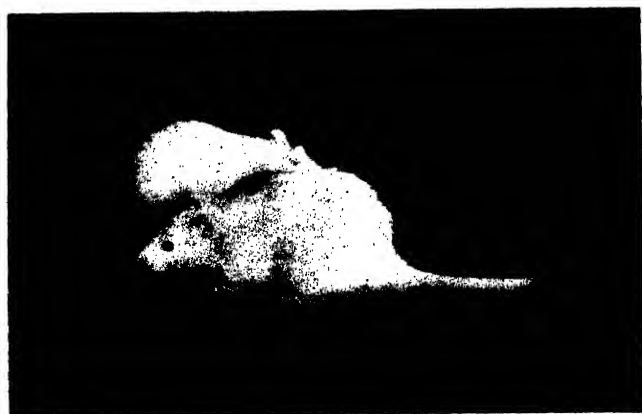


FIG. 1.

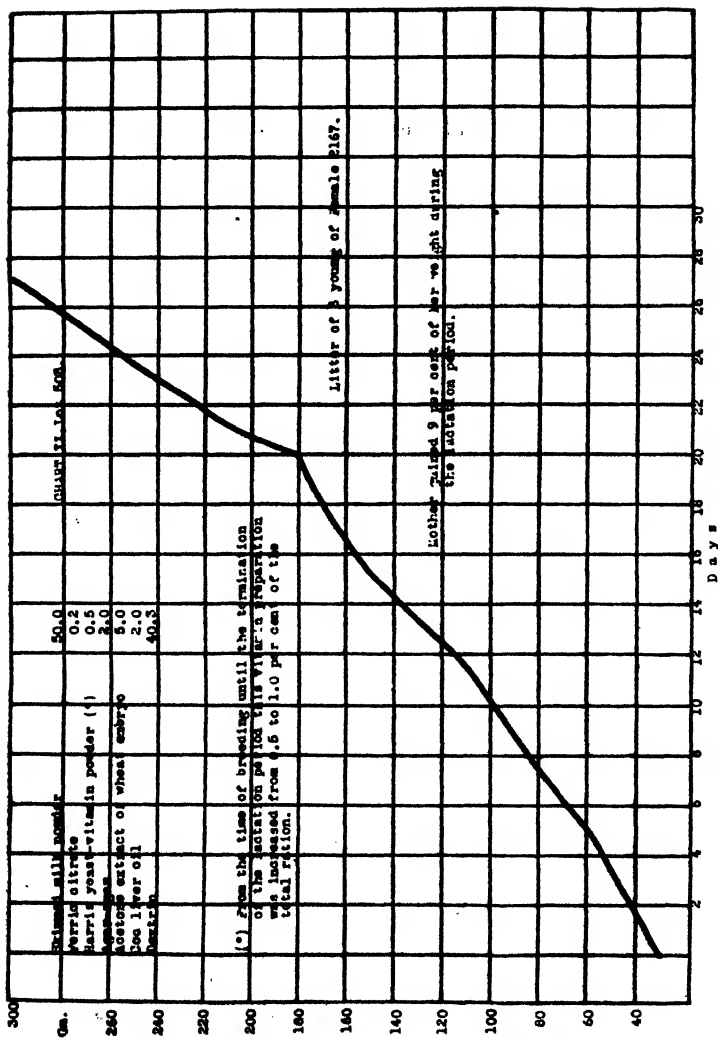


CHART II.

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weaned on the 23rd day after birth. The mother of this litter had a skimmed milk powder ration containing a 5 per cent acetone extract of wheat embryo (litter of Female 2167, Chart II, Lot 503).

CHARTS IV and V, Lot 504. Female 2173 had ten young, and was given six, weighing 29 gm., to rear. The addition of 5 per cent of a benzene (benzol) extract of the wheat embryo resulted in fertility and successful lactation. The individual young, however, did not attain the weight of 40 gm. (which is the minimum weight we secure with young reared on a diet composed of natural food stuffs) until the 26th day.

Female 2172 had ten young, and was given six, weighing 28 gm., to rear. She lost two on the 2nd day, and the remaining four young collectively

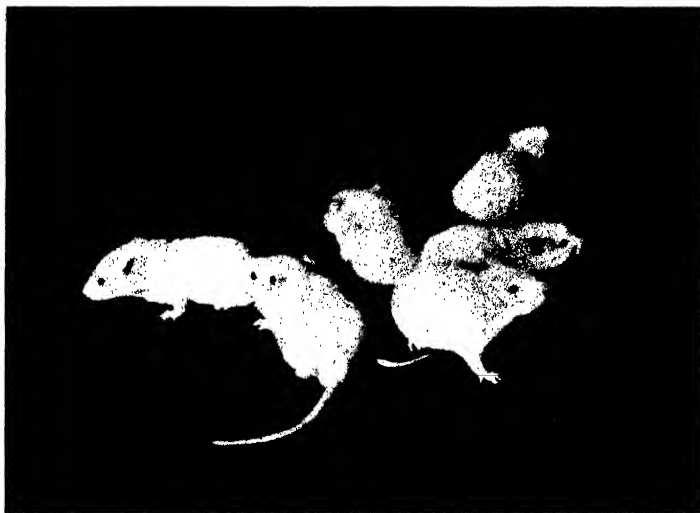


FIG. 2.

weighed 21 gm. They were successfully weaned on the 20th day, and on the 22nd day of lactation weighed 40 gm. each. As she had fewer young, there was a smaller burden on the mammary gland of Mother 2172, and, instead of maintaining her weight as Female 2173 with a litter of six, she gained 17 per cent of her body weight during the lactation period.

Female 2174, Lot 504, had ten young, and was given six, weighing 29 gm., to rear. The entire litter was reared normally until the 21st day, at which time there was an insufficient milk secretion and one young died. The remaining five young, however, were successfully weaned on the 24th day, and on the 26th day collectively weighed 228 gm., or 45.6 gm. each.

The mothers of the above litters received a 5 per cent benzene (benzol) extract of the wheat embryo in addition to their basal skimmed milk powder ration.

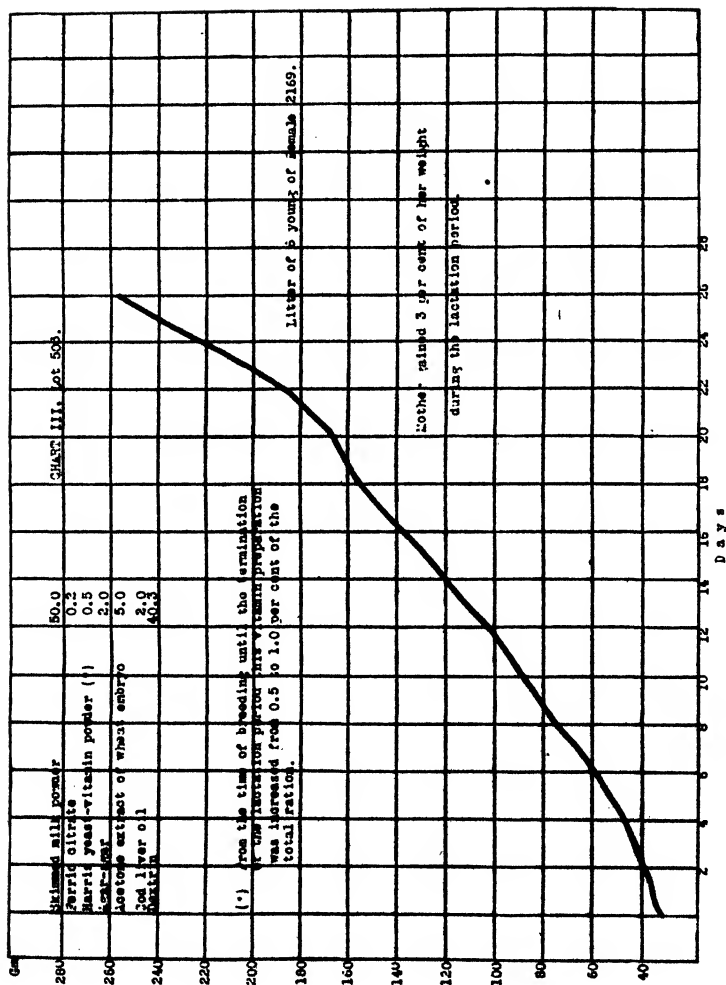


CHART III.

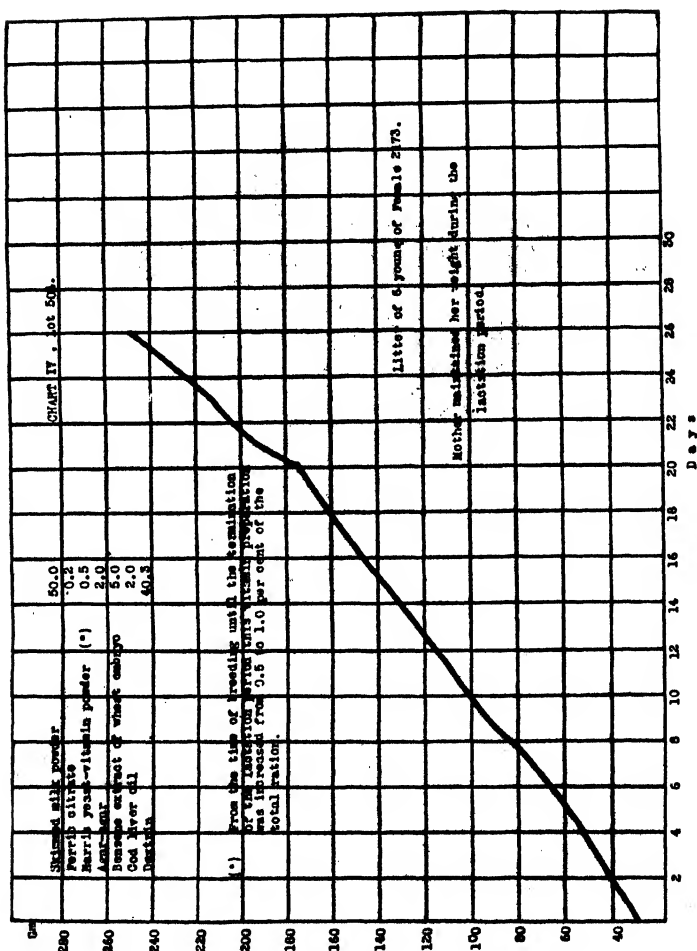


CHART IV.

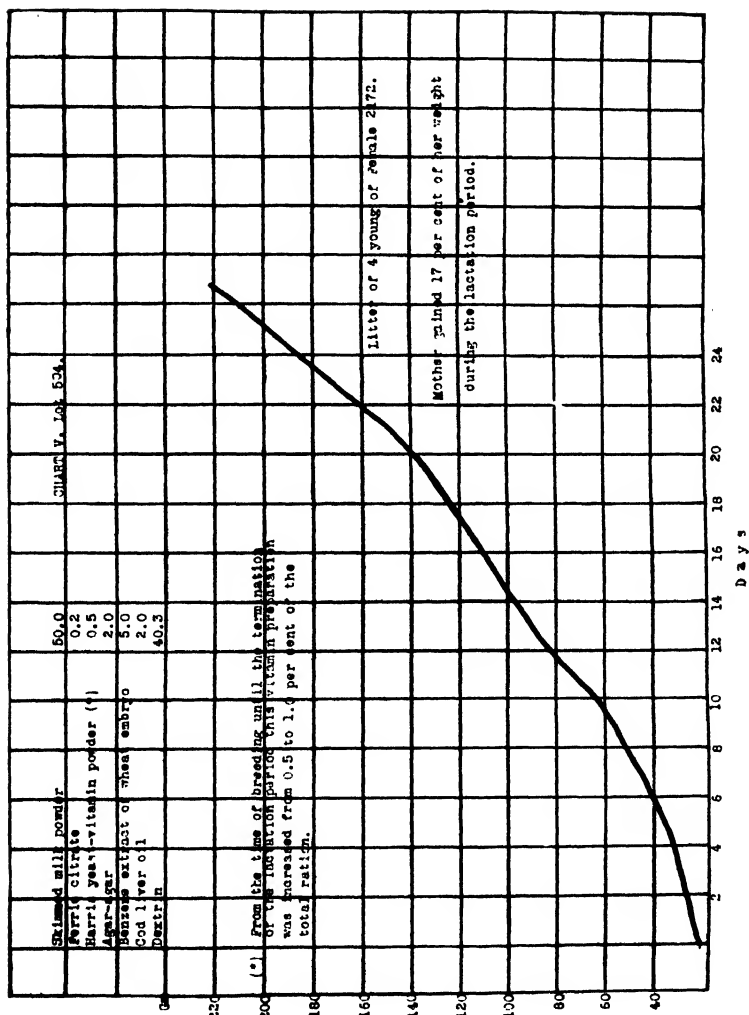


CHART V.

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The above experiments clearly demonstrate that vitamin E, the organic complex indispensable for fertility and lactation, is extracted with acetone and benzene² as well as ether.

Recently Evans³ stated that "lactation is always seriously impaired on 'pure' diets. The average weaning weight of the animals resulting from such lactations is almost exactly half that which is normal, *i.e.*, 20 grams instead of 40 grams on the twenty-first day of life."

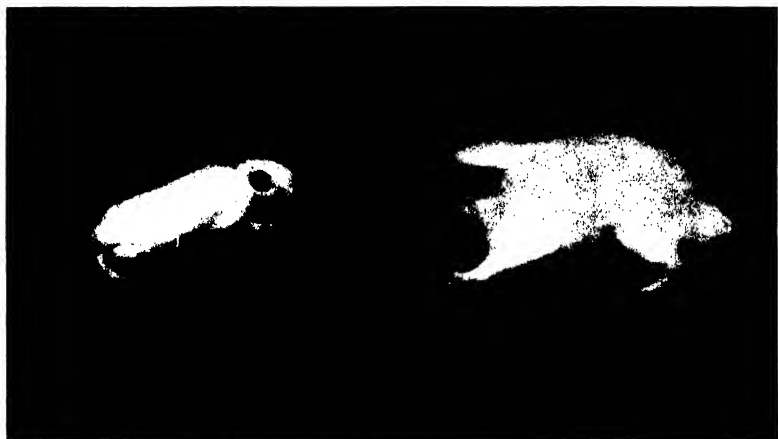


FIG. 3.

Fig. 3 shows a small animal, 13 days old, reared on a skimmed milk powder diet fortified with a 5 per cent ethereal extract of the wheat embryo. On his "pure casein diets" Evans' young animals attain that weight at the end of the 21st day which marks the termination of the lactation period. The larger animal is one young of a litter of six, 22 days old, weighing 42 gm., which has been reared and weaned on a skimmed milk powder ration containing 5 per cent of a benzene extract of the wheat embryo.

² The acetone and benzene (benzol) extracts of the wheat embryo were prepared by extraction in a large Soxhlet apparatus by allowing about 1,000 gm. of wheat embryo to extract for 14 hours until the solvent siphoned twelve to fourteen times.

³ Evans, H. M., *Science*, 1924, ix, 20.

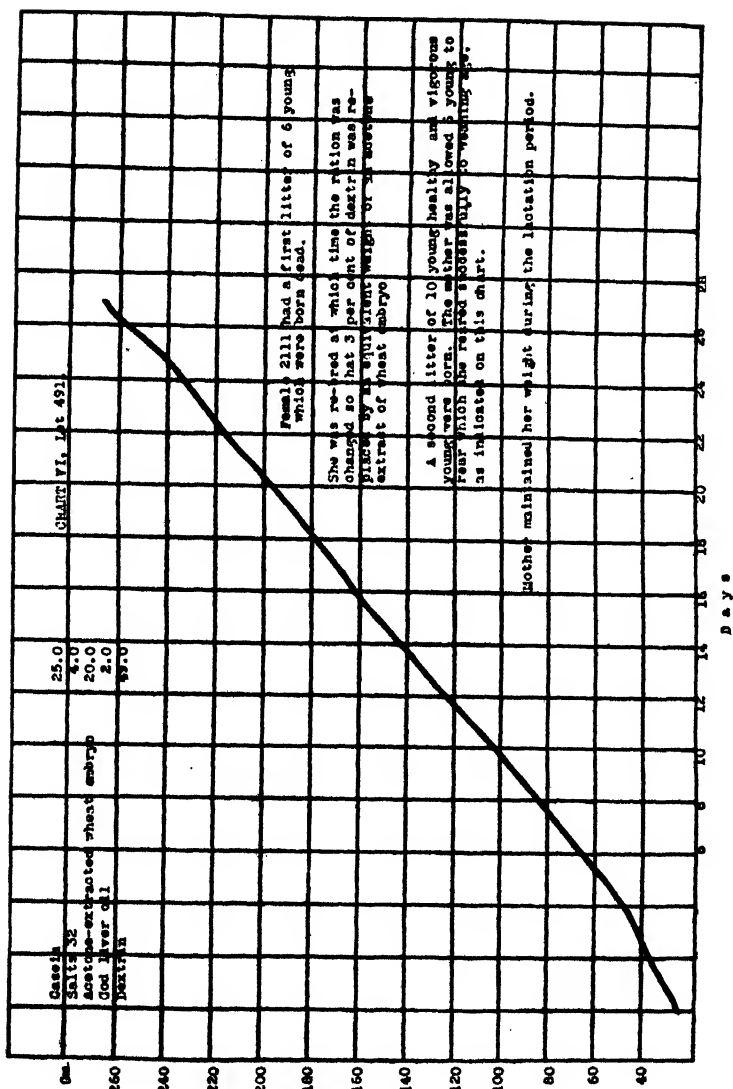


CHART VI.

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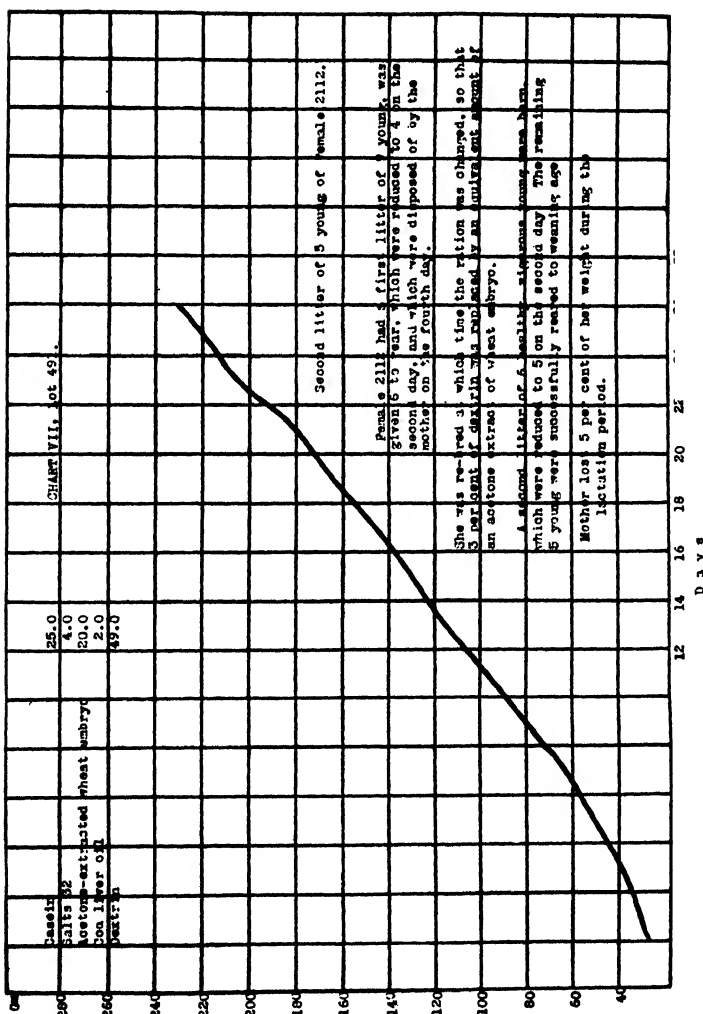


CHART VII.

Since wheat germ oil extracted with ether from wheat embryo contains an organic complex essential for reproduction, it was anticipated that a fat-free preparation of wheat embryo should be inadequate even for fertility; consequently, the following experiments were initiated to test this point.

Lot 485 (control experiment) received the following ration: casein, 25; salts 32, 4; whole wheat embryo, 20; cod liver oil, 2; and dextrin, 49. On the above ration 100 per cent fertility was secured and three females, Nos. 2082, 2083, and 2084, each reared one litter of six healthy, vigorous young successfully throughout the lactation period, and the young were weaned on the 25th day after birth.

Lot 486 had the same ration as Lot 485, with the exception that the wheat embryo had been extracted for 7 hours with ether.

Female 2087 had ten young, six of which were born dead, and the remaining four died on the 2nd day after delivery. Female 2088 was found with only two young which died later in the day. Female 2089 had 7 young, all of which were born dead.

Charts VI and VII, Lot 491, are self-explanatory. On a ration containing 20 per cent acetone-extracted wheat embryo, fertility was secured, but lactation was an absolute failure. The addition of an acetone extract of the wheat embryo to the ration resulted in successful lactation with the second litters.

Lot 492 received the following ration: casein, 25; salts 32, 4; agar-agar, 2; whole wheat embryo, 15; cod liver oil, 2; and dextrin, 52. On this ration two females had eight and eleven young, respectively, and reared litters of six each subnormally for a period of 15 days. The failure in rearing of young during the latter part of lactation may be ascribed to a reduction of the concentration of wheat germ oil; also to a reduction in the possible supplementary amino acids wheat embryo may supply to casein in this ration.

An inspection of Table I shows that wheat embryo when extracted with ether for 7 hours and used in such large quantities as 1,000 gm. at a time still leaves a residual amount of fat to the extent of 0.45 per cent. After being extracted for 14 hours with hot acetone, 0.22 per cent of residual wheat oil is left in the wheat embryo. Whole wheat embryo, it will be noted, contains 14.7

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per cent of fat. Lot 486, which had 20 per cent of ether-extracted, and Lot 491, which had 20 per cent of acetone-extracted, wheat embryo, had, then, 0.09 and 0.044 per cent of wheat germ oil, respectively, in their rations, which amounts were sufficient for fertility, but inadequate for lactation. The control lot received 20 per cent of whole wheat embryo, or 2.94 per cent of wheat germ oil in the ration. That amount evidently was sufficient for both fertility and lactation.

Since neither 7 hours of ether extraction nor 14 hours of hot acetone extraction removed the fat from wheat embryo completely, a further attempt was made to exhaust the germ of wheat of all its oil as follows: Whole wheat embryo was extracted for 7 hours with ether in a Soxhlet apparatus until the solvent siphoned six to eight times. After the ether had been removed by allowing the material to be exposed to the air for 4 hours in

TABLE I.
Fat in Wheat Embryo Preparations.

Whole wheat embryo.	Wheat embryo extracted 7 hrs. with ether.	Wheat embryo extracted 14 hrs. with hot acetone.	Wheat embryo extracted 7 hrs. with ether; 3 days with carbon tetrachloride; 14 hrs. with hot acetone; and 14 hrs. with hot benzene.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
14.7	0.45	0.22	0.07

flat pans, it was subjected to 3 days extraction with carbon tetrachloride in the cold. After the carbon tetrachloride had been expelled from the wheat embryo by gently heating on a steam bath for several hours, the preparation was further subjected to extraction with hot acetone in a Soxhlet apparatus for 14 hours until the solvent siphoned twelve to fourteen times. After the acetone had been driven off by a similar procedure, the wheat embryo was finally extracted for 14 hours with hot benzene. The preparation thus treated was found to contain 0.07 per cent of residual fat.

Lot 490 had the same ration as Lot 492, with the exception that it was given the wheat embryo containing the 0.07 per cent residual fat. On such a low fat-containing diet, in which only 0.01 per cent of wheat germ oil had been introduced into the

ration, Female 2107 gave birth to two dead young 3 days prematurely, and later had nine young, of which six were born dead, and the other three were disposed of on the 3rd day. Female 2106 did not even become pregnant. Female 2108 had six young, all of which were born dead.

From food consumption records and the amount of residual wheat germ oil contained in the ration of Lot 490, it is calculated that as little as 1 mg. of wheat oil per rat per day is sufficient for fertility. Considerably more of that oil, however, is necessary for successful lactation. Just what the minimum amount necessary for lactation is, will be determined later.

In this connection the recent work of Mattill, Carman, and Clayton⁴ is of interest. They found that wheat embryo after being extracted for 24 to 36 hours with anhydrous ether loses its power to produce fertility. They state that of twelve females only one at 93 days gave birth to a litter unseen. Evidently these investigators have obtained a completely fat-free wheat embryo preparation with their procedure, and the conclusion of Evans³ that "wheat embryo in the fat-free condition, is as effective in its galactogogic action as the whole wheat embryo, and that, it would appear, therefore, that the food material necessary for mammary function is not soluble in fats" may have been formed on the basis of results of experiments in which he employed wheat embryo material extracted with ether containing small but appreciable amounts of residual fat, as indicated in the above experiments.

SUMMARY.

Benzene (benzol) and acetone, as well as ether, extract the organic complex, designated as vitamin E, essential for reproduction. It is, then, concluded that the reproductive dietary factor is a fat-soluble vitamin.

⁴ Mattill, H. A., Carman, J. S., and Clayton, M. M., *J. Biol. Chem.*, 1924, lxi, 729.

THE DISTRIBUTION OF NITROGEN IN THE PROTEIN FRACTION OF TUBERCLE BACILLI AFTER REMOVAL OF TUBERCULINIC ACID.

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(Received for publication, January 14, 1925.)

In the course of research in this laboratory on the chemistry of tubercle bacilli it became necessary, before undertaking the study of experimental conditions and analytical methods to be utilized for the separation, purification, and identification of the different protein fractions of this organism, to acquire a knowledge of the amino acid composition of the protein residue obtained after thorough defatting of the bacilli and removal of the nucleic acid (tuberculinic acid)^{1,2} with water or dilute alkali. At present we have practically no knowledge of the intermediate changes which nitrogen undergoes in the natural synthesis of protein from nitrogen compounds of known constitution, and also of the essential α -amino acid combinations which are preferred as building units by bacteria in the synthesis of their cell protein. A careful study of the problem forces one to inquire whether, in their normal course of cell development, certain amino acid combinations are destroyed by bacteria during their growth, leading to the production of organic combinations of the nature of "protamines." Ruppel³ claims to have separated from tubercle bacilli cells by extraction with 1 per cent sulfuric acid a characteristic protein combination called "tuberculo-seamine"—and it was classed by him among the protamines. The analytical data thus far published do not permit one, however, to draw very definite conclusions regarding its constitution. Tamura,⁴ in a

¹ Johnson, T. B., and Brown, E. B., *J. Biol. Chem.*, 1922, liv, 721.

² Brown, E. B., and Johnson, T. B., *J. Biol. Chem.*, 1923, lvii, 199.

³ Ruppel, W. G., *Z. physiol. Chem.*, 1898-99, xxvi, 218.

⁴ Tamura, S., *Z. physiol. Chem.*, 1913, lxxxvii, 85.

later paper, has commented on this observation of Ruppel's, and he actually endeavored to obtain a protamine fraction by treating defatted tubercle bacilli at a low temperature with strong sulfuric acid (2 parts of concentrated H_2SO_4 and 1 part H_2O), but without success. He writes as follows: "Niemals aber bin ich auf einen proteinartigen Körper gestossen, der mit den aus Fischsperma gewonnenen Protaminen einige Ähnlichkeit gezeigt hätte." From what is known at the present time regarding the action of strong sulfuric acid on proteins, it is questionable whether Tamura would be expected to obtain a compound possessing the properties of Ruppel's tuberculoseamine by working under the conditions recorded in his paper.

The most detailed examination of the cleavage products of the protein present in tubercle bacilli, previous to the work in this laboratory, is that reported by Tamura.⁴ The analytical results published by him, however, are so widely divergent from those recently published by Johnson and Brown,⁵ that it was important to pay further attention to this phase of our problem and to compare more critically the experimental technique employed in the processes of preparation and purification of the respective protein fractions of tubercle bacilli which were used for amino acid analysis.

The investigations both of Tamura and of Johnson and Brown were conducted with tubercle bacilli that had been grown under normal conditions on sterile beef bouillon with addition of the necessary inorganic salts and glycerol to promote growth.⁶ Working with 70 gm. of bacilli which had been dried *in vacuo* at 37° , Tamura defatted his material by extraction with ether and alcohol to constant weight. His bacilli suffered a loss of 27.1 per cent by this treatment, leaving 51 gm. of material which still contained an unknown percentage of lipoids. These defatted bacilli were then subjected to an acid treatment by trituration with 66 per cent sulfuric acid, followed by dilution with water until the proportion of free acid was reduced to 5 per cent. Under these conditions an amorphous precipitate was obtained which

⁵ Johnson and Brown,¹ p. 729.

⁶ It is our plan to follow up this research with an examination and analysis by our method of the protein fraction of tubercle bacilli which has been grown on artificial media of known composition.

was separated by filtration, washed with water, dried at 37°, and finally extracted again with ether and alcohol to remove lipid material. After drying at 37° in a vacuum 29.2 gm. of a powder were obtained, containing 9.2 per cent of nitrogen and 0.7 per cent of phosphorus. This gave all the reactions characteristic of protein and also a test for tryptophane, but did not respond to a test for sulfur. By hydrolysis of 28 gm. of this crude protein residue with 25 per cent sulfuric acid, Tamura obtained the analytical results which are recorded in Table I. In other words, the 29.2 gm. of protein, obtained after treatment with sulfuric acid and subjected to amino acid analysis, represented only 41.8 per cent of the original dried tubercle bacilli or 57.3 gm. of his defatted bacilli. Tamura does not state in his paper the temperature nor the time of treatment with strong sulfuric acid before diluting with water. He does write, however, that this acid filtrate obtained after filtration of his protein gave no biuret test, and did not respond to a Millon's test. These observations are quite remarkable when one considers that 31.1 per cent of his original bacilli was extracted during the acid treatment. Throughout his process of purification Tamura worked entirely with strong acid reagents and no alkali was brought into contact with his material.

The protein fraction analyzed by Johnson and Brown was obtained from tubercle bacilli in an entirely different manner. In this case, the bacilli, after drying in vacuum at 37°, were defatted by extraction with hot toluene and, after freeing from this organic solvent, were then triturated at ordinary temperature with dilute sodium hydroxide solution (3 to 5 per cent) to remove nucleic acid (tuberculinic acid). They recovered 84 per cent of the original defatted bacilli which gave on analysis 10.2 per cent of nitrogen and 1.19 per cent of phosphorus, and did not respond to the color tests characteristic of uracil and thymine after hydrolyzing, indicating that this phosphorus is held in the form of other combinations besides nucleic acid. When this protein fraction (84 gm.) was analyzed by Johnson and Brown, according to the Van Slyke method, for its distribution of nitrogen, the analytical results recorded in Table I were obtained. Therefore, to summarize, the protein fraction analyzed by Tamura represented only 57.2 per cent of his defatted bacilli, while the analysis reported

TABLE I.

Protein.....	Gliadin.		Protein of tubercle bacilli.					Spinach.	Alfalfa.	<i>Zea mays</i> .
	Osborne, Van Slyke, Leavenworth,* and Vinograd.	Coghill.	Tamura.††	Johnson and Brown.§	Coghill.	Coghill.	Coghill.	Chibnall.¶	Chibnall.¶	Chibnall.¶
Amide N ₂	24.61	24.45		11.83	13.50	10.23		6.93	5.51	7.44
Humin N ₂	0.58	1.05	15.79	4.11	6.72	6.66		2.47	2.72	4.57
Arginine.....	5.45	4.69	7.64	10.63	10.28	12.21	13.18	13.80	15.32	14.69
Cystine.....	0.80	0.75		1.26	Trace.	Trace.	Trace.	1.27	0.84	0.77
Histidine.....	3.39	4.35	1.53	11.48	9.13	13.81¶	14.06	3.89	3.09	4.70
Lysine.....	1.33	1.60	1.49	3.69	7.21	3.69¶	6.44	9.63	9.97	8.78
Amino N ₂	51.95	51.36	66.59	47.39	52.10	50.30		58.09	58.56	55.81
			NH ₄ = 1.15							
Non-amino N ₂	10.70	9.54	Unknown N = 6.0	9.34	3.88	3.10		2.58	3.19	2.04
Total.....	98.81	97.79	100.19	99.73	102.82	100.00		98.66	99.20	99.80

* Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, 259.

† Tamura, S., *Z. physiol. Chem.*, 1918, lxxxvii, 85.

‡ Nitrogen distribution not determined by Van Slyke procedure.

§ Johnson, T. B., and Brown, E. B., *J. Biol. Chem.*, 1922, liv, 721.

¶ Total basic nitrogen determination lost. Calculated by difference.

|| Chibnall, A. C., *J. Biol. Chem.*, 1924, lxi, 303. Chibnall, A. C., and Nolan, L. S., *J. Biol. Chem.*, 1924, lxii, 173, 179.

by Johnson and Brown is based on a protein fraction free from nucleic acid and representing 84 per cent of their original defatted bacilli.

In the light of these entirely different experimental procedures employed for purification of their protein fractions,^{1,2,4} it might be expected, therefore, to find that the analytical data expressing the quantitative distribution of nitrogen in the respective protein fractions would not be in agreement. In fact, Johnson and Brown's method of operating by defatting with toluene and then extracting the fat-free bacilli with dilute alkali at ordinary temperature is to be recommended if one desires to avoid hydrolytic changes in the protein. Treatment of protein with sulfuric acid of 60 to 70 per cent strength even at ordinary temperature, as employed by Tamura, involves an experimental condition which is very favorable for rapid and deep seated changes in the protein molecule with formation of polypeptide and cyclic anhydride combinations. Many of these simple combinations have been shown to give none of the tests characteristic of proteins. For example, the protein elastin undergoes hydrolysis with 70 per cent sulfuric acid at ordinary temperature, giving the anhydride of the polypeptide glycyl-*L*-leucine.⁷ Also the fibroin of silk, when subjected to the action of 70 per cent sulfuric acid at 0°, is broken down with the formation of the anhydride of *D*-alanyl-glycine.⁸ In fact, when fibroin is treated with 3 parts of 70 per cent sulfuric acid at 0° solution begins almost immediately and is practically complete in 2 hours and, after standing 4 days at 26°, hydrolysis is complete and dilution with water produces no precipitate of protein. Furthermore, fibroin dissolves in cold concentrated hydrochloric acid very readily, and, if the solution is not allowed to stand too long, dilution with alcohol results in the precipitation of *sericoïn*, described by Weyl.⁹ Exposure to the action of strong hydrochloric acid for 24 hours gave a solution of modified protein which yielded no precipitate when diluted with alcohol. Furthermore, the interesting data obtained

⁷ Fischer, E., and Abderhalden, E., *Ber. chem. Ges.*, 1906, xxxix, 2315.

⁸ Fischer, E., and Abderhalden, E., *Ber. chem. Ges.*, 1906, xxxix, 752.

⁹ Weyl, T., *Ber. chem. Ges.*, 1888, xxi, 1407.

by Abderhalden and coworkers¹⁰ as a result of their recent investigations on the molecular structure of proteins affords quite conclusive proof that interaction of protein with strong sulfuric acid at ordinary temperature leads to complete destruction of molecular structure. They have shown that such treatment is very favorable for the formation of diacipiperazine combinations.

It seems very probable, therefore, in the light of all these facts that Tamura was dealing with a highly modified protein fraction of tubercle bacillus, and that the analytical results obtained by him do not represent a correct approximation of the distribution of nitrogen in the protein of this organism. We have now repeated our work, using bacilli of a different source, and publish in this paper (Table I) distribution values for nitrogen obtained by the Van Slyke method, which are quite in accord with those previously obtained by Brown. In carrying out our purification of the protein fraction used for analysis we have followed the procedure of Johnson and Brown and analyzed a fraction which had been freed from nucleic acid (tuberculinic acid). When one considers that the analytical results represent values obtained by analyses of different units of bacteria and that the best methods of purification of our bacterial protein have not been developed, the agreement in analytical determinations is quite remarkable. An inspection of Table I reveals the fact that both Brown and Coghill are in close agreement in their analytical determinations, which disclose the large percentage of the basic amino acids and the low proportion of cystine in their protein fractions. It is questionable whether the figures obtained for cystine by application of the Van Slyke method really approach an accurate approximation of the true value. For the sake of comparison we have recorded in Table I the nitrogen distribution values obtained by Chibnall with the three cell proteins isolated from the cytoplasm of the

¹⁰ Abderhalden, E., *Z. physiol. Chem.*, 1923, cxxviii, 119; cxxix, 106; cxxx, 205; cxxxi, 284. Abderhalden, E., and Stix, W., *Z. physiol. Chem.*, 1923, cxxix, 143; 1924, cxxxii, 238. Abderhalden, E., and Klarman, E., *Z. physiol. Chem.*, 1923, cxxix, 320; 1924, cxxxv, 199; cxxxix, 64. Abderhalden, E., and Komm, E., *Z. physiol. Chem.*, 1924, cxxxiv, 113, 121; cxxxvi, 134; cxxxix, 147, 191; cxi, 99. Abderhalden, E., Klarman, E., and Komm, E., *Z. physiol. Chem.*, 1924, cxi, 92. Abderhalden, E., Klarman, E., and Schwab, E., *Z. physiol. Chem.*, 1924, cxxxv, 180. Abderhalden, E., and Schwab, E., *Z. physiol. Chem.*, 1924, cxxxix, 68, 169.

leaves of spinach, alfalfa, and *Zea mays* (ensilage corn). Here also we are dealing with a high content in hexone bases and a low percentage in cystine as we observed in the case of our tubercle bacillus protein. All of these proteins exhibit a widely different distribution of nitrogen than does gliadin from wheat.¹¹

SUMMARY.

1. The work of Johnson and Brown on the analysis of protein from tubercle bacilli has been repeated and confirmed.

2. Application of the Van Slyke method for determination of nitrogen distribution in the protein has revealed a high percentage of the hexone bases and a very low content in cystine.

3. The protein analysis reveals a very close relationship to the cell proteins separated by Chibnall from the cytoplasm of the leaves of spinach, alfalfa, and ensilage corn.

¹¹ This work was done with the aid of a grant from the National Tuberculosis Association. We take this opportunity to express our appreciation of their cooperation and support of the research.

THE RÔLE OF VITAMINE B IN RELATION TO THE SIZE OF GROWING RATS.*

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WITH THE COOPERATION OF HELEN C. CANNON.

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(Received for publication, January 20, 1925.)

In studies of nutrition wherein the individual dietary factors are to be controlled as far as possible, vitamine concentrates are essential. As a rule the vitamine-bearing products obtained directly from natural sources contain a large number of known and unknown ingredients. The success of certain experiments depends on employing as a source of vitamine B a product free from the particular food factor being studied. Until this vitamine is available in a state of chemical purity ideal conditions for such experiments are unattainable. We have, however, a yeast concentrate¹ which is free from protein and wholly free from substances soluble in alcohol or ether. Furthermore, as a much smaller amount of this product suffices for the normal growth of a young rat than of any other available product it is possible by its use to conduct experiments under conditions more nearly approaching the ideal than with yeast or other sources of vitamine heretofore employed. Since we are using this yeast concentrate for many of our feeding experiments it has become important for us to know how much is needed daily by growing rats of various sizes, fed under the conditions prevailing in our laboratory. We have accordingly made a series of experiments, similar to those previously made with brewery yeast,² in which definite

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1922, liv, 739.

quantities of this concentrate were supplied daily to groups of young rats of like age and size. The feeding of the various groups was begun at different ages and continued until growth ceased. That failure to continue to grow was due to an insufficiency of vitamine B was shown by the resumption of growth when the daily dose of the yeast concentrate was increased.

Since the experiments to be described were conducted chiefly for our own guidance the conditions employed were like those prevailing in most of our investigations in which vitamine B was not the special subject of study. Consequently, no attempt was made to prevent the rats from eating their feces which, we have already pointed out, may be a disturbing factor in such experiments.³ Evidence has already been furnished² of a correlation between the size of the animal and its vitamine B requirement for growth or maintenance. We soon found that daily doses of the yeast concentrate which were adequate as sources of vitamine B for rats under 100 gm. of body weight no longer sufficed when the animals attained a larger size. Our earlier comparative tests indicated the daily requisite of vitamine B per 100 gm. of body weight to approximate what is contained in 50 to 60 mg. of our dry yeast. Further evidence that the rate of growth, under otherwise comparable dietary and environmental conditions, depends upon the vitamine dosage is afforded by our similar studies of the yeast concentrate.⁴ A "standard" food mixture, consisting of casein 18, starch 54, lard 15, butter fat 9, and salt mixture⁵ 4 per cent, and demonstrated to promote growth to adult size when an abundance of vitamine B in the form of yeast is supplied, was furnished *ad libitum* along with tap water to the animals. The yeast concentrate was fed daily, apart from the rest of the ration; the rats being kept in individual cages. Some of the results are summarized in Charts 1 and 2, which show the changes in body weight of animals thus started at sizes of approximately 50, 80, 150, and 250 gm.

³ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Pub. No. 156*, pt. 2, 1911, 61. Steenbock, H., Sell, M. T., and Nelson, E. M., *J. Biol. Chem.*, 1923, lv, 399.

⁴ For convenience we have employed the product made essentially according to the directions of Osborne and Wakeman¹ and supplied by The Harris Laboratories, Tuckahoe, N. Y.

⁵ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

The results in Series A with a vitamine B dosage of 40 mg. of the yeast concentrate per day, show that this quantity sufficed in many instances, though not for all the animals, to induce growth to a body size of 300 gm. or above, on the selected diets. At any rate it sufficed as a rule to maintain a satisfactory condition above 200 gm. over long periods of time. This agrees with our experience when this amount of the yeast concentrate was used in a large number of other experiments made for other purposes. That this dosage was near the minimum for the larger rats is shown by the resumption of growth in several tests when the amount of vitamine concentrate was increased. On a dose of 30 mg. per day (Series B) it has been exceptional to carry the animals above 200 gm. without increasing the vitamine B intake. With 20 mg. per day the limit of good gains was usually reached still earlier (Series C); and when the test was made with animals of a larger initial size (Group 4) decline in weight was a rule. The results with 10 mg. per day (Series D) are interesting merely as showing how the rate of growth of smaller animals also may be decreased when the vitamine B supply is curtailed. As in our experience with whole yeast² we have observed an occasional rat to continue to grow to comparatively large size on diets containing a supply of vitamine B far below the minimum for most animals of comparable size. This was conspicuously true, for example, of one rat in Series C, Group 1; likewise in Series B, Group 3, of our study of yeast.² Perhaps the animals in these exceptional cases were coprophagists and thus secured a greater supply of vitamine B than did their companions.

Within admittedly wide variations the minimum effective dose of the yeast concentrate here used to produce growth at approximately normal rate may be placed at 15 to 20 mg. per 100 gm. of body weight in contrast with 50 mg. for the yeast itself.⁶ The organic part of the concentrate that we have used constituted not more than 0.4 per cent of the average food intake of our experimental animals. As we have remarked in an earlier com-

⁶ Comparable results with regard to the dosage of such concentrates have been secured in unpublished investigations by Dr. A. H. Smith and Dr. G. R. Cowgill. When precautions are taken, by means of suitable cages, to prevent access to the feces, their rats required a notably larger dosage of vitamine B.

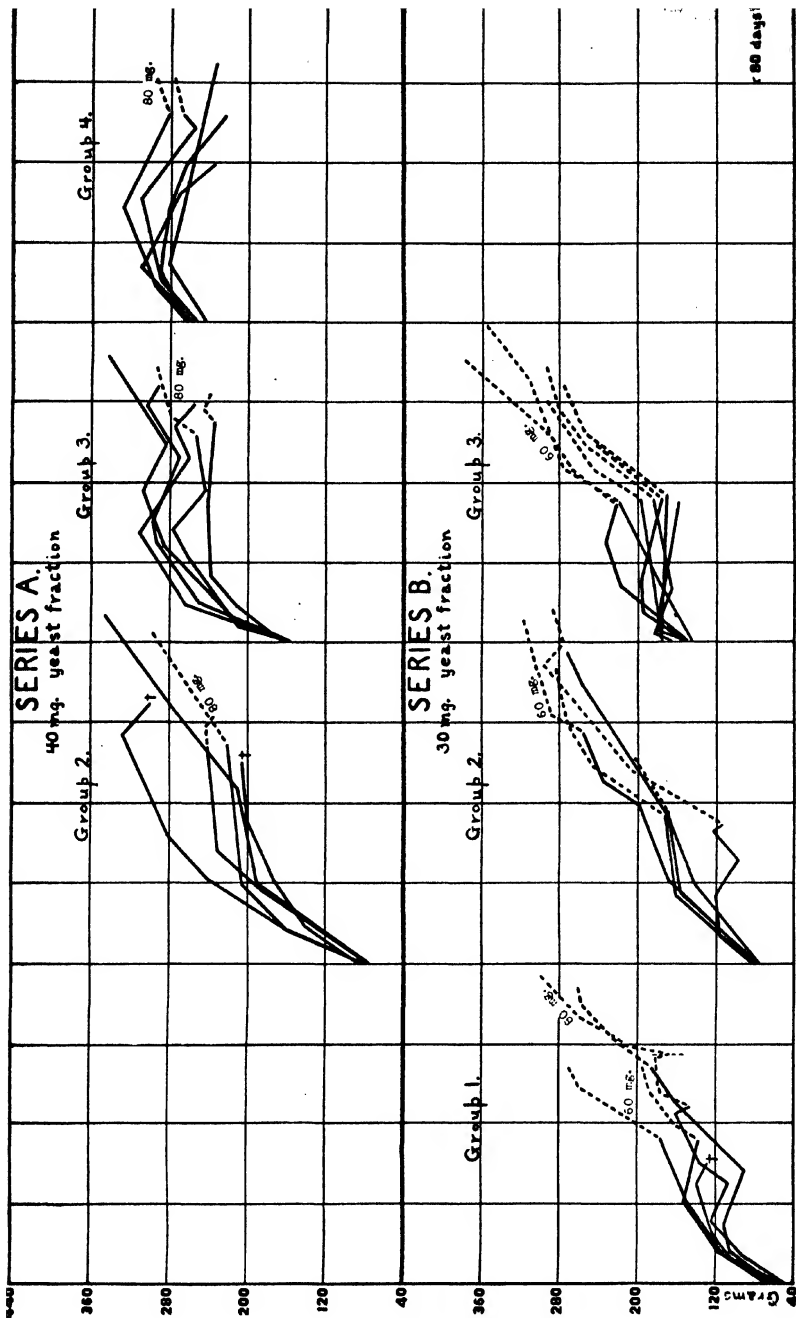


CHART 1.

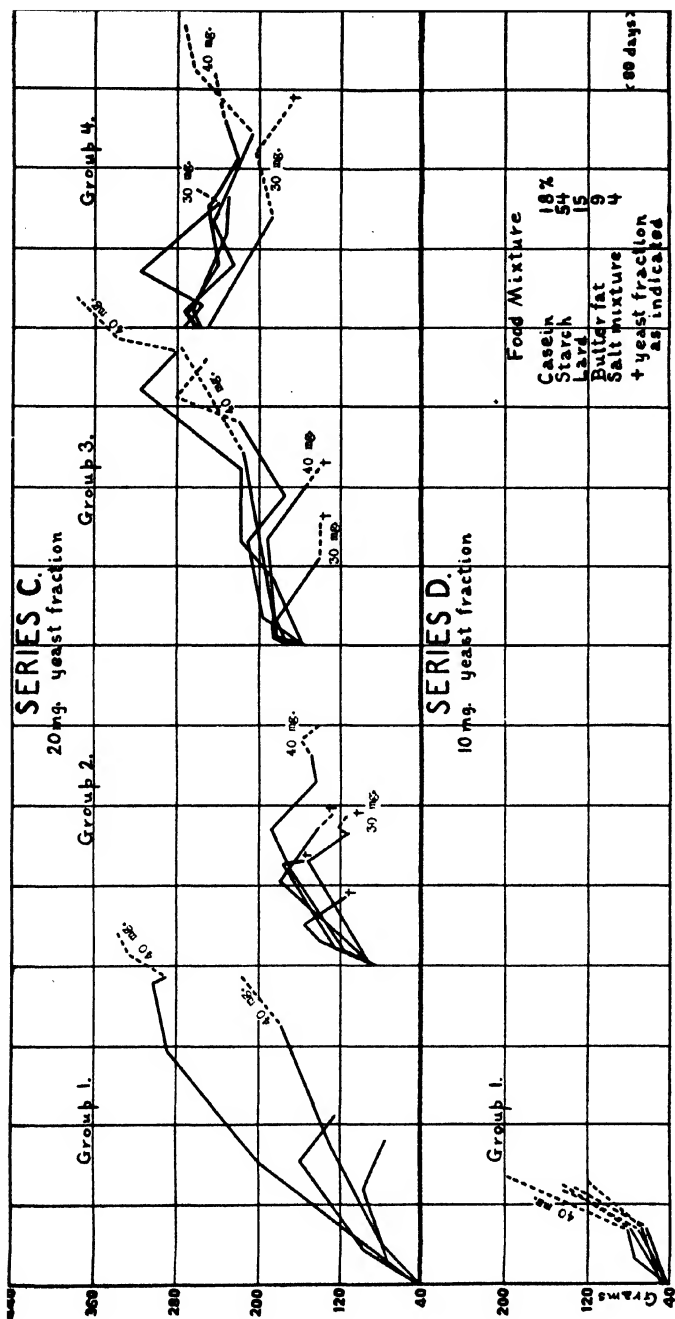


CHART 2.

CHARTS 1 and 2. These charts show changes of body weight of rats receiving an otherwise adequate diet supplemented by various fixed quantities of vitamin B furnished by a yeast concentrate.

That cessation of growth or gains below the expected average for the size of the rat employed were in most cases actually due to insufficient intake of vitamin B is indicated on the graphs by the broken lines showing a resumption of growth when the daily dose was increased

munication, it is little short of surprising to see how the seemingly small variations in the intake of a product, which at best must be composed in large part of extraneous matter other than the vitamine *per se*, are attended by corresponding changes in the body weight of the animals. When the smaller doses are fed to larger animals a decline in weight ensues; with intermediate doses there may be maintenance at various levels of body weight; with the larger intake for the smaller animals growth ensues.

THE DISTRIBUTION OF THE UNSATURATED FATTY ACIDS, CHOLESTEROL, AND CHOLESTEROL ESTERS IN EXPERIMENTAL ANEMIA.*

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(Received for publication, January 3, 1925.)

Much discussion exists in the literature which attempts to correlate blood destruction in anemic conditions with changes in the blood lipoids. Attention has been directed more especially to cholesterol and the unsaturated fatty acids because of the antihemolytic properties of the former and the alleged hemolytic action of the latter. With regard to cholesterol, there is more or less general agreement that anemias are usually associated with low blood cholesterol values (1, 2, 3, 4, 5). On the other hand, the significance of the unsaturated fatty acids as a factor in anemia is still a matter of controversy.

Faust and Tallquist (6) attribute bothriocephalus anemia to the oleic acid arising from the cholesterol oleate present in the proglottis of the parasite. In clinical anemias Eppinger (7) and King (8) have reported high iodine numbers as well as increases in the total fat of the blood. High plasma fat in anemia has been found by Bloor and MacPherson (9) who do not, however, regard their observations as certain evidence that lipid abnormalities are a cause of anemia. Gibson and Howard (10) have recorded low blood and plasma fat values in pernicious anemia. It appears that when the fat content is diminished, the unsaturated fatty acid fraction is proportionately greater, though in total not exceeding that found normally. Of more direct bearing to the present problem are the observations of Joannovics and Pick (11) in the case of experimental toluylenediamine anemia. In sub-

* A brief report of these observations was presented at the Washington meeting of the American Society of Biological Chemists, December, 1924.

acute intoxication with toluylenediamine, injury to the liver is accompanied by fatty changes and a marked increase of the unsaturated fatty acid content of the liver. It is postulated that the unsaturated fatty acids, chiefly oleic, are responsible for the blood destruction and for the hemolytic properties of the liver extracts.

Two considerations cast doubt on the pathogenetic significance of oleic acid. This compound, as well as fatty acids of greater unsaturation, is formed normally in fat metabolism. There is no convincing data to show that the content of these substances in anemic conditions is much in excess of the physiological limits. Secondly, the evidence adduced by Faust (12), pointing to the production of anemia on continued feeding of oleic acid to dogs, has not been confirmed by other investigators (13).

In the present work an attempt has been made to study the nature of the blood lipid changes which occur in experimental acute anemia with particular reference to the distribution between the plasma and corpuscles of the unsaturated fatty acids, cholesterol, and cholesterol esters. Of the methods which the writer has employed for producing experimental anemia (14), two have been selected. It is to be recalled that acetylphenylhydrazine produces rapid red cell destruction without appreciable injury to the liver. On the other hand, the initial effects of poisoning with symmetrical diisopropylhydrazine hydrochloride are extensive fatty and destructive changes in the liver accompanied by anhydremia. Subsequently, anemia develops. As the fatty changes in the liver produced by this method are more severe than those caused by toluylenediamine, it should be possible to demonstrate even more convincingly the effect of unsaturated fatty acids on blood destruction, assuming that the hypothesis of Joannovics and Pick (11) with regard to toluylenediamine anemia is correct.

EXPERIMENTAL.

Dogs were employed as the experimental animals. Symmetrical diisopropylhydrazine anemia was studied in Dogs 2, 6, 7, and 14. Lipoid changes during the early stages of diisopropylhydrazine poisoning were followed in Dogs 3A and 5A, and of 2, 2'-azobispropane intoxication in Dog 4A. Acetylphenylhydrazine

anemia was produced in Dogs 9, 12, 13, 17, and 18. Dogs 6, 7, 9, and 13 were made anemic following splenectomy. The effect of toluylenediamine poisoning on the unsaturated fatty acid content of the blood was studied in Dogs 20 and 21. A number of animals included in this series were kept under observation for long periods in connection with a study of the effect of splenectomy on red cell regeneration in experimental anemia (15).

In a number of experiments, determinations were made of the total fatty acids and lecithin, as well as of cholesterol, cholesterol esters, and the unsaturated fatty acids. Cholesterol and total fatty acids were determined by the method of Bloor, Pelkan, and Allen (16). The method of Bloor and Knudson (17) was used for cholesterol esters. Iodine numbers were determined by a micro modification of the Hanus method, essentially similar to that employed by Gibson and Howard (10). This method has been found very reliable, although it is to be appreciated that by this technique, the determinations are made without the preliminary removal of cholesterol and its esters.

The results obtained were calculated in terms of millimolar equivalents of oleic acid in 100 cc. of blood, plasma, or corpuscles. Several considerations make this mode of expression desirable. Extraction of the saturated fats from the corpuscles by the alcohol-ether mixture is admittedly incomplete, whereas, from the uniformity of the results, it is believed that the unsaturated fats are removed somewhat more completely. Iodine number calculations on the basis of the total fat content would, therefore, be subject to an error in the case of the whole blood and corpuscles. Another error may be introduced in the determination of the total fatty acids, owing to the limitations of nephelometric procedure. Furthermore, any physiological significance which the unsaturated fatty acids may have in blood destruction would be determined more clearly by comparing the variations in the total concentration of the unsaturated fatty acids than by a comparison of the changes in the iodine absorption values of the blood fat.

That alterations in the size of the individual corpuscle may be a factor in determining lipoid equilibria suggested itself early in the present work. To bring out this relationship, the volume of the individual corpuscle was calculated from the red cell count and the corpuscle volume determined, always under uniform

conditions, by centrifuging the oxalated blood. While the results are regarded only as close approximations, they serve, nevertheless, as a satisfactory basis for further discussion. The average normal value obtained for the volume of one corpuscle in fourteen dogs was 6.5×10^{-8} c.mm., the extreme variations being from 5.58×10^{-8} to 7.65×10^{-8} . It was likewise indicated that the hemoglobin concentration might be another factor. For purposes of comparison, therefore, the hemoglobin content was calculated similarly for the individual corpuscle from the hemoglobin values obtained by the Newcomer (18) method. The variations in the fourteen dogs were from 1.26×10^{-8} to 2.22×10^{-8} mg. of hemoglobin, the average being 1.84×10^{-8} mg. per corpuscle. In this connection it may be recalled that in certain pathological conditions studied by Csonka (19) the iodine numbers, representing the unsaturated fatty acids, were higher than normal, especially in cases with low hemoglobin values.

The data obtained in the four dogs with symmetrical diisopropylhydrazine anemia (Table I) will now be considered. In Dog 2, the anemia was accompanied by moderate increases in the volume and hemoglobin content of the individual corpuscle; no significant changes occurred either in the content or distribution of the unsaturated fatty acids; the total cholesterol was diminished, but its distribution, likewise, was not affected. The outstanding change was the appearance of a relatively large amount of cholesterol esters in the corpuscles. The cholesterol in the corpuscles of normal dogs is present entirely or almost entirely as free cholesterol. It should be pointed out that the presence of cholesterol esters in the corpuscles is not a constant feature in experimental anemia.

Following splenectomy, a secondary anemia usually develops which reaches its greatest severity in from 3 to 6 weeks. Associated with the postsplenectomy anemia in Dog 6, cholesterol esters were found in the corpuscles on February 5, but not on January 30. The red cell count of this animal on the day of the operation (December 14) was 8.6 million. In Dog 7, a similar interval had elapsed after the operation and was accompanied by a reduction in the red cell count from about 6 to 4.3 million. Cholesterol esters were consistently present in the corpuscles of this dog before it was made anemic with symmetrical diiso-

TABLE I.

Unsaturated Fatty Acids, Cholesterol, and Cholesterol Esters in Experimental Anemia.

Protocols.—Dog 2. Female, weight 7.8 kilos. Injected 180 mg. symmetrical diisopropylhydrazine hydrochloride Feb. 10, 1924; 200 mg. Feb. 20; 350 mg. Mar. 11; 300 mg. Mar. 18.

Dog 6. Male, weight 6.0 kilos. Splenectomy Dec. 14, 1923. Injected 200 mg. symmetrical compound Feb. 13, 1924; 200 mg. Feb. 20; 300 mg. Mar. 12; 300 mg. Mar. 18.

Dog 7. Male, weight 7.2 kilos. Splenectomy Dec. 14, 1923. Injected 200 mg. symmetrical compound Feb. 13, 1924; 200 mg. Feb. 20; 300 mg. Mar. 12; 300 mg. Mar. 18.

Dog 14. Male, weight 11.5 kilos. Injected 170 mg. symmetrical compound Apr. 19, 1924; 200 mg. Apr. 23; 250 mg. May 2; 200 mg. May 9.

Dog No.	Date.	Red count in millions per c. mm.	Corpuscle volume $n \times 10^{-3}$ c. mm.	Hemoglobin in corpuscle $N \times 10^{-3}$ mg.	Total cholesterol in 100 cc.			Cholesterol esters in 100 cc.			Unsaturated fatty acids in millimols of oleic acid per 100 cc.		
					Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.
	1924		n	N	mg.	mg.	mg.	mg.	mg.	mg.	mm	mm	mm
2	Feb. 10	6.40	6.25	1.98	222	213	235	78	138	0.0	2.50	2.33	2.80
	Mar. 24	3.84	6.75	2.52	177	162	219	73	76	62	2.26	2.12	2.64
	Apr. 5	4.00	6.75	2.16	164	140	193				2.50	2.34	2.87
6	Jan. 30	6.24	5.61	1.69	145	160	118	80	136	-0.0*			
	Feb. 5	5.80	5.52	1.76	160	160	160	93	111	59	2.40	1.97	3.30
	Mar. 25	3.60	7.22	2.22	128	112	173	77	77	77	2.35	1.92	3.50
7	Feb. 1	4.30	6.97	2.00	182	145	267	84	92	67	2.07	1.83	2.64
	" 5	4.32	6.95	1.95	182	135	283	68	82	37			
	Mar. 29	2.32	9.05	1.99	150	105	319	55	54	57	2.13	1.64	3.99
	Apr. 8	2.98	8.05	1.94	150	105	292	57	54	67	1.94	1.66	2.84
14	" 14	6.20	6.77	1.74	217	196	246	94	164	-0.0	2.72	2.24	3.40
	" 18	6.40	6.41	1.65	202	194	212	101	166	7	2.82	2.68	3.02
	May 21	4.00	8.00	2.24	182	225	97	108	160	0.0	2.47	2.54	2.32

* The cholesterol ester content of the corpuscles was calculated from the data obtained on the plasma and whole blood. Because of the limitation of the method, values slightly less than 0.0 were obtained in a number of cases.

propylhydrazine hydrochloride. However, in both cases, a further change in the distribution of the cholesterol esters occurred. Before the severer form of anemia was produced, the greater proportion of the esters was present in the plasma. With the production of the anemia, the esters became about equally distributed between the plasma and corpuscles and in Dog 7 on April 8, the concentration of the esters in the corpuscles even exceeded that in the plasma.

As in the case of Dog 2, Dog 6 showed a reduction in total cholesterol; both the corpuscular volume and hemoglobin content were increased, but no appreciable change was noted either in the concentration or distribution of the unsaturated fatty acids. Similar changes in the volume and hemoglobin content of the individual corpuscle occurred in Dog 14.

Of the four dogs in this group, an alteration in the distribution of the unsaturated fatty acids was manifested in Dog 7 on March 29. Accompanying the marked increase of the unsaturated fatty acid concentration of the corpuscles, was a marked increase in the volume of the individual corpuscle without any increase of its hemoglobin content.

The total fat in the plasma was not affected in Dog 6. In the anemic condition, this animal showed, however, an increase in the fat of the whole blood from the normal value of 0.282 to 0.400 per cent. There was likewise, a moderate increase in the lecithin, from 0.434 to 0.469 per cent in the plasma and in the whole blood from 0.408 to 0.464 per cent. On the other hand, in Dog 7, the lecithin values were not changed appreciably from the normal during the progress of the anemia.

The relationship between corpuscle volume, hemoglobin content, and unsaturated fatty acids is indicated more clearly in the data outlined in Table II (acetylphenylhydrazine anemia). In Dog 9, the size of the corpuscle was increased, while the hemoglobin content diminished. A definite change in the distribution of the unsaturated fatty acids occurred, the concentration in 100 cc. of corpuscles being more than doubled. In Dog 12, on February 19, the unsaturated fatty acids were distributed normally despite the severity of the anemia and the increased size of the corpuscles. At this time the hemoglobin content was above the initial value. Subsequently, the hemoglobin content decreased

TABLE II.

Unsaturated Fatty Acids, Cholesterol, and Cholesterol Esters in Experimental Anemia.

Protocols.—Dog 9. Male, weight 7.1 kilos. Splenectomy Dec. 21, 1923. Injected 250 mg. acetylphenylhydrazine Feb. 10, 1924; 200 mg. Feb. 20; 200 mg. Feb. 26; 400 mg. Mar. 7.

Dog 12. Male, weight 5.3 kilos. Injected 240 mg. acetylphenylhydrazine Feb. 10, 1924; 200 mg. Feb. 20; 200 mg. Feb. 25.

Dog 13. Male, weight 11.5 kilos. Splenectomy Mar. 25, 1924. Injected 300 mg. acetylphenylhydrazine Apr. 4, 1924; 300 mg. Apr. 18; 400 mg. Apr. 21; 400 mg. Apr. 23.

Dog 17. Female, weight 9.7 kilos. Injected 500 mg. acetylphenylhydrazine Oct. 17, 1924.

Dog 18. Male, weight 14.7 kilos. Injected 500 mg. acetylphenylhydrazine Oct. 22, 1924; 450 mg. Oct. 27.

Dog No.	Date.	Red count in millions per c. mm.	Corpuscle volume $n \times 10^{-3}$ c. mm.	Hemoglobin in corpuscle $N \times 10^{-3}$ mg.	Total cholesterol in 100 cc.			Cholesterol esters in 100 cc.			Unsaturated fatty acids in millimols of oleic acid per 100 cc.		
					Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.
	1924		n	N	mg.	mj.	mg.	mg.	mj.	mg.	mm	mm	mm
9	Jan. 28	6.40	7.74	2.22	191	160	222	52	101	0.0	1.86	2.06	1.67
	Feb. 19	2.88	8.69	2.00	154	148	180	62	60	70	2.34	1.90	4.00
	Mar. 1	2.00	9.00	2.00	174	182	139	60	80	—0.0	2.32	2.03	3.65
	" 11	1.48	10.80	1.89	187	148	394	78	91	25	2.29	2.13	3.16
12	Feb. 6	6.72	6.70	2.17	160	148	176	55	101	0.0	2.04	2.02	2.05
	" 10	6.72	6.70	2.17	148	126	175	36	73	0.0	2.30	2.22	2.38
	" 19	2.80	8.94	2.54	156	143	196	80	85	64	2.36	2.39	2.27
	Mar. 1	1.95	9.25	1.62	191	167	300				2.35	1.82	4.75
13	" 24	4.80	6.67	1.98	160	154	172	60	94	—0.0	2.02	1.91	2.30
	Apr. 3	5.20	6.94	1.88	167	151	194	36	60	3	2.01	1.64	2.66
	" 9	2.08	9.60	2.21	116	106	155	43	63	0.0	1.79	1.77	1.87
	" 18	3.69	7.62	2.18	140	125	179	35	58	1	2.00	1.72	2.56
	" 24	1.70	9.71	1.88	123	116	158	49	67	—0.0	1.86	1.63	3.04
	" 26	0.82	11.00	1.83	133	124	211	46	49	37	1.77	1.69	2.80
17	Oct. 15	6.08	6.42	1.76	244	210	298	120	200	0.0	2.87	2.71	3.13
	" 20	4.40	6.14	1.33	219	185	343	129	159	23	2.66	2.38	3.69
18	" 21	5.28	6.80	1.85	133	118	190	51	80	—0.0	2.45	2.07	3.12
	" 28	2.20	7.28	1.59	186	180	220	78	83	50	2.85	2.72	3.85

very markedly while the volume continued to increase. These changes were accompanied by a very noticeable increase in the unsaturated fatty acid content of the corpuscles.

Essentially similar results were obtained in the following experiment (Dog 13). Fairly uniform distribution was manifest throughout the progress of the anemia as long as the increased size of the corpuscle was accompanied by a similar change in its hemoglobin content. However, with the diminution of the hemoglobin concentration on April 24, an accumulation of unsaturated fatty acids occurred in the corpuscles.

Acute intoxication with acetylphenylhydrazine in Dogs 17 and 18 was followed by relatively insignificant variations of the corpuscular volume, but with appreciable depletion of the hemoglobin content of the individual corpuscle. In both experiments these manifestations were accompanied by moderate increases in the unsaturated fatty acid content of the corpuscles.

If attention is now directed to the changes in the plasma and whole blood, it will be observed that the unsaturated fatty acid concentrations were maintained within remarkably uniform and narrow limits. The physiological significance of these observations assumes a special interest when regarded in the light of a suggestion advanced by Bloor (20) with respect to the rôle of the red corpuscles in fat metabolism. It is pointed out that during fat absorption an increase in lecithin occurs in the blood and is most marked in the corpuscles where the fat content is likewise increased. The inference is drawn that fat is taken up by the corpuscles and transformed into lecithin. According to Bloor, this assumption is not invalidated by his own observations which show that the increase of lecithin in the corpuscles cannot always be demonstrated, in view of the probability that the lecithin readily passes from the corpuscles to the plasma. The corpuscles may be concerned possibly with fat metabolic changes other than the formation of lecithin and cholesterol esters such as desaturation of the fatty acids. It may be shown by calculation from the data of Dog 7 that the increases in lecithin and cholesterol esters are equivalent to but a small portion of the increase in unsaturated fatty acids.

If the assumption is correct that the red corpuscles play an

important if not an essential part in fat metabolism, it follows that by reducing the number of corpuscles very markedly, the share of the burden on the individual corpuscle might be increased. The capacity of the corpuscle for lipid transformations may then be determined by a variety of factors such as the volume of the corpuscle and the content of its non-lipoid constituents. This is indicated from the data presented in the present paper. In the anemic condition, the red cells were or were not increased in volume; the hemoglobin content remained unchanged or was either increased or decreased. The combination of circumstances most effective in increasing the unsaturated fatty acid content of the corpuscle was an increase in its volume together with a decrease of its hemoglobin content. There may be other determining factors such as alterations in water distribution between the plasma and corpuscles. This will constitute the subject of a forthcoming investigation.

In all the animals in the acetylphenylhydrazine group, cholesterol esters were absent from the normal corpuscles and were usually present in the corpuscles of the anemic animals. Exception to this occurred on March 1, in Dog 9, when no esters were observed in the corpuscles, and in Dog 13. In the latter animal, the corpuscles remained practically free from cholesterol esters until April 26, when the red cell count had fallen to 820,000.

Effect of Splenectomy.

According to King (8) and Eppinger (7), the removal of the spleen results in an increase of cholesterol and a decrease of the unsaturated fatty acids of the blood. Bloor and MacPherson (9) obtained irregular changes in the fatty acids following splenectomy, but the cholesterol was increased in nearly all their cases. Dubin and Pearce (21) observed that after splenectomy the blood of dogs shows practically no change from the normal in the amount of total fat and unsaturated fatty acids. In our experiments, splenectomy did not affect appreciably either the total cholesterol or the unsaturated fatty acids. As pointed out previously, postsplenectomy anemia was frequently associated with the presence of cholesterol esters in the corpuscles.

Effect of Liver Injury.

The development of lipemia in hydrazine poisoning has been observed by Underhill and Baumann (22). Symmetrical diisopropylhydrazine and the corresponding azo compound, 2, 2'-azobispropane, bear some resemblance to hydrazine in that they all cause very extensive fatty and degenerative changes in the liver (23). The chemical nature of these changes will be studied in another connection.

The data outlined in Table III present definite evidence that, even during the onset of the intoxication when the liver involvement was most severe and in progress, the concentration and distribution of the fatty acids in the blood remained unchanged. Moderate concentration of the blood occurred in all cases. A small amount of hemoglobin destruction was indicated, but the volume of the individual corpuscle was not affected.

These observations, therefore, show that excessive concentration in the blood of the unsaturated fatty acids does not occur during the early stages of symmetrical diisopropylhydrazine anemia and hence cannot be regarded as a factor in the blood destruction which follows. In addition, the distribution data lend further support to the view suggested with respect to the factors determining unsaturated fatty acid distribution between the plasma and corpuscles.

Toluylenediamine Anemia.

In two experiments (Table IV), subacute toluylenediamine poisoning resulted in changes similar to those observed in other forms of anemia. Contrary to the observations of Joannovics and Pick (11), the unsaturated fatty acids of the blood and plasma were not affected in either experiment. A moderate increase in the corpuscles in Dog 20 was concomitant with a similar increase in the corpuscular volume without a corresponding increase of the hemoglobin content. The cholesterol values obtained in Dog 20 were the lowest observed in any of the normal dogs in this series. Examination of all the data presented indicates that in the normal animal, low cholesterol concentrations are generally associated with low concentrations of the unsaturated fatty acids, whereas higher values for cholesterol are accompanied by greater concentrations of the unsaturated fatty acids.

TABLE III.

Unsaturated Fatty Acids, Cholesterol, and Cholesterol Esters during the Early Stages of Poisoning with Symmetrical Diisopropylhydrazine and 2, 2'-Azobispropane.

Protocols.—Dog 3 A. Female, weight 8.0 kilos. Injected 450 mg. symmetrical diisopropylhydrazine hydrochloride Aug. 15, 1924.

Dog 4 A. Female, weight 9.7 kilos. Injected 0.4 cc. 2, 2'-azobispropane.

Dog 5 A. Female, weight 11 kilos. Injected 400 mg. symmetrical diisopropylhydrazine hydrochloride Sept. 13, 1924.

Dog No.	Date.	Red count in millions per c. mm.	Corpuscle volume $n \times 10^{-3}$ c. mm.	Hemoglobin in corpuscle $N \times 10^{-3}$ mg.	Total cholesterol in 100 cc.			Cholesterol esters in 100 cc.			Unsaturated fatty acids in millimols of oleic acid per 100 cc.		
					Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.
	1924		n	N	mg.	mg.	mg.	mg.	mg.	mg.	mm	mm	mm
3 A	Aug. 15	6.32	6.95	1.76	140	120	166	63	116	0.0	2.02	1.88	2.19
	" 21	8.64	6.95	1.67	143	133	150	51	89	25	2.01	2.06	1.97
4 A	Sept. 10	6.56	5.58	1.26	160	136	202	50	78	0.0	2.47	2.18	2.96
	" 12	7.44	5.28	1.25	160	128	208	51	84	0.0	2.36	2.02	2.90
5 A	" 13	6.16	5.95	1.62	228	256	153	118	164	38	3.20	3.30	3.05
	" 16	6.80	5.90	1.57	206	242	152	109	180	3	(lipemia) 3.02	3.37	2.51

TABLE IV.

Unsaturated Fatty Acids and Cholesterol in Toluylenediamine Anemia.

Protocols.—Dog 20. Male, weight 16.4 kilos. Injected 1.0 gm. toluylenediamine Nov. 10, 1924; 1.0 gm. Nov. 12.

Dog 21. Male, weight 8.8 kilos. Injected 1.0 gm. toluylenediamine Nov. 18, 1924.

Dog No.	Date.	Red count in millions per c. mm.	Corpuscle volume $n \times 10^{-3}$ c. mm.	Hemoglobin in corpuscle $N \times 10^{-3}$ mg.	Total cholesterol in 100 cc.			Unsaturated fatty acids in millimols of oleic acid per 100 cc.		
					Blood.	Plasma.	Corpuscles.	Blood.	Plasma.	Corpuscles.
	1924		n	N	mg.	mg.	mg.	mm	mm	mm
20	Nov. 10	5.92	5.97	1.61	85	105	58	1.63	1.27	2.22
	" 12	3.20	7.82	1.80	91	64	172	1.50	1.29	2.10
	" 13	2.10	9.00	2.04	100	63	257	1.48	1.18	2.71
21	" 18	6.80	6.70	2.06	174	167	182	1.74	1.64	1.85
	" 25	2.40	6.55	1.88	177	174	191	1.56	1.60	1.36

SUMMARY.

The greater proportion of the cholesterol present in the plasma is in the form of esters. In ten normal dogs the variations observed ranged from 57 to 96.5 per cent. The variations in the whole blood were between 31 and 55 per cent. In all cases, the corpuscles of the normal dogs were free from the esters of cholesterol.

In the anemic state, a reduction in the cholesterol ester: total cholesterol ratio occurred in the plasma. This ratio was nearly always increased in the blood due to the appearance, usually, of cholesterol esters in the corpuscles.

Severe liver injury occurs during the early stages of symmetrical diisopropylhydrazine anemia. At the height of the intoxication no change was observed in the unsaturated fatty acids of the blood. This indicates that the unsaturated fatty acids are not concerned in the blood destruction which follows the early stages of the intoxication.

No significant changes in the cholesterol and unsaturated fatty acid content of the blood were observed following splenectomy. Cholesterol esters were present in the corpuscles of dogs made mildly anemic as a result of splenectomy.

The concentration of the unsaturated fatty acids in the plasma was maintained within very uniform and narrow limits in anemias due to acetylphenylhydrazine, symmetrical diisopropylhydrazine hydrochloride, and toluylenediamine. On the other hand, an increased concentration in the corpuscles was observed frequently. Two factors have been studied which appear to determine the increased accumulation of the fatty acids; namely, the volume and hemoglobin content of the individual corpuscle. An increase in the volume of the individual corpuscle with a decrease or without a corresponding increase of its hemoglobin content was associated, in our experiments, with an increased content of the unsaturated fatty acids.

The presence in the corpuscles of larger amounts of unsaturated fatty acids as well as of cholesterol esters is taken to mean that the corpuscles are concerned with fat metabolism. In view of the fewer number of red cells in anemia, the individual corpuscle may assume a proportionately greater share in lipid transformations than the normal corpuscle.

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Addendum.—After the present paper went to press, there appeared a contribution by Bloor (Bloor, W. R., *J. Biol. Chem.*, 1925, lxiii, 1) on the plasma lipoids in experimental anemia. Bloor finds an increase in the percentage and degree of unsaturation of the fatty acids in experimental anemia produced by hemorrhage. One of the suggestions offered by Bloor is that the diminished number of corpuscles may be the cause of this phenomenon since the corpuscles presumably take part in certain phases of fat metabolism. This is quite in accord with one of the writer's conclusions arrived at in this paper. Another point emphasized by Bloor is with regard to the presence in the plasma of fatty acids having more than one double bond, the iodine number of the unsaturated fatty acids averaging 173 in the anemic dogs. This value is nearly twice that of the iodine number of oleic acid (90). Calculating from the data in our paper in which the unsaturated fatty acids are expressed in terms of millimols of oleic acid, values are obtained which are about twice as high as the values found by more direct methods for determining the unsaturated fatty acid fractions of the blood. In other words, these data point to the presence in the blood of unsaturated fatty acids of more than one double bond in the proportions defined by Bloor.

THE INFLUENCE OF INSULIN AND EPINEPHRINE ON THE LACTIC ACID CONTENT OF BLOOD AND TISSUES.*

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Briggs, Koechig, Doisy, and Weber (1) recently put forward the theory that insulin influences the reversible reaction $\text{glucose} \rightleftharpoons \text{lactic acid}$ in the direction of lactic acid and that glucose is formed in diabetic animals because the influence of insulin is lacking. This theory seemed welcome in one respect, because it might have had some bearing on the decrease in the liver and muscle glycogen that is observed in the normal fasting animal after the administration of insulin. Since, on the other hand, some recent facts seemed to contradict such a conception, it was thought advisable to extend the observations of the authors mentioned above by including lactic acid determinations in tissues. Epinephrine, which in some instances may be regarded as an antagonist of insulin, was used for comparative purposes. While this work was in progress, there appeared a communication by Tolstoi, Loebel, Levine, and Richardson (2), who studied the influence of insulin on the lactic acid content of the blood of diabetic patients and made observations on the lactic acid content of the blood of normal persons following epinephrine. Our results and conclusions seem to be in harmony with their findings.

EXPERIMENTAL.

1. *Lactic Acid in Blood.*

The changes in the sugar and lactic acid content of the blood following the injection of insulin or epinephrine were studied on

* Presented before the American Society of Biological Chemists in Washington, D. C., December, 1924.

rabbits, cats, and on one dog. The animals were bled from the marginal ear vein and enough blood for duplicate sugar and lactic acid determinations was easily and quickly obtained. Generally not more than 2.5 cc. of blood were taken, so that repeated blood sampling did not cause any serious loss of blood. Special precautions were used to prevent muscular movements of the animals before and during the collection of blood, since this might have influenced the lactic acid content of the blood. The rabbits were placed in wooden baskets, which were just large enough to allow them a squatting position, but which were too small for any movement, and they remained perfectly quiet. The cats were made pets before they were used for experiments. They were held on the lap during the blood collection and purring was taken as a sign that the animals were at ease during the procedure. Some cats, which would not sit quietly enough on the lap, were enclosed in a sack, which had a slit just large enough for the head and a slit parallel to the back of the animals, the latter serving as an opening through which injections could be made. The cats did not, as one would suppose, try to escape from the sack, but behaved very quietly, apparently because they felt themselves protected in their enclosure.

The blood sugar was determined by the Hagedorn and Jensen (3) method. For the analysis of the lactic acid content of the blood, Clausen's (4) permanganate method, which is a micro adaptation of the von Fürth-Charnass procedure, and the H_2SO_4 method were used. Later on, in the analysis of the lactic acid in tissues, preference was given to the H_2SO_4 method, which required less attention, allowed more determinations to be run at one time, and gave better results in our hands than the permanganate procedure. Clausen's method was not used for experiments until considerable time had been spent in checking up the different steps of the method and in analyzing zinc lactate solutions with a known content of lactic acid. The recovery of lactic acid was the same as reported by Clausen and amounted to 90 per cent in the case of the permanganate procedure and to 98 to 100 per cent with the H_2SO_4 procedure. The method, however, when applied to filtrates from biological material was not very accurate, in that duplicate determinations were sometimes 5 or even 10 per cent apart. It also happened, especially

TABLE I.

Influence of Insulin and Epinephrine on the Lactic Acid Content of the Blood of Rabbits.

Rabbit No.	Blood sugar.	Lactic acid.	Remarks.
	mg. per 100 cc.	mg. per 100 cc.	
1	127	22.6	Before insulin. Fasted for 18 hrs.
	50	32.3	90 min. after 20 units insulin. 95 min. after insulin. Symptoms of hypoglycemia. 5 gm. glucose subcutaneously.
	70	24.2	20 min. later.
1	123	31.8	Before insulin. Fasted for 5 hrs.
	59	31.4	120 min. after 20 units insulin.
			175 " " insulin. Symptoms of hypoglycemia. 4 gm. glucose subcutaneously.
	75	36.9	15 min. later.
1	107	27.6	Before insulin. Fasted for 17 hrs.
			120 min. after 16 units insulin. Very violent convulsions.
	43	106.5	3 min. after convulsive seizure. 5 gm. glucose subcutaneously.
	87	17.5	120 min. later.
2	105	35.7	Before insulin. Fasted for 48 hrs.
	55	43.8	165 min. after 14 units insulin.
	47	45.1	240 " " insulin.
2	98	21.5	Before insulin. Fasted for 19 hrs.
	100	25.5	" "
	54	28.0	155 min. after 12 units insulin.
	48	20.2	240 " " insulin.
3	106	14.2	Before insulin. Fasted for 18 hrs.
	107	13.1	" "
			120 min. after 18 units insulin. Violent convulsions.
	43	56.6	2 min. after convulsive seizure. 5 gm. glucose subcutaneously.
			120 min. later strong convulsion.
	40	51.7	3 min. after convulsive seizure.

256 Insulin and Epinephrine on Lactic Acid

TABLE I—*Concluded.*

Rabbit No.	Blood sugar.	Lactic acid.	Remarks.
	mg. per 100 cc.	mg. per 100 cc.	
3	111	29.3	Before insulin. Fasted for 17 hrs. 65 min. after 18 units insulin. Violent convulsions.
	38	95.4	2 min. after convulsive seizure.
4	125	32.7	Before insulin. On the 3rd day of phlorhizin poisoning, D:N ratio 2.78.
	66		120 min. after 12 units insulin.
	63	24.8	240 " " insulin.
5	133	14.8	Before epinephrine. Fasted for 17 hrs.
	117	15.2	" "
	332	94.6	155 min. after 0.7 mg. epinephrine.
	336	54.8	250 " " epinephrine.
5	95	23.3	Before epinephrine. Fasted for 17 hrs.
	94	16.2	" "
	304	113.4	120 min. after 1.0 mg. epinephrine.
	402	78.5	240 " " epinephrine.
6	102	39.7	Before epinephrine. Fasted for 48 hrs.
	274	50.1	75 min. after 1.0 mg. epinephrine.
	204	71.5	235 " " epinephrine.
7	98	17.2	Before glucose. Fasted for 40 hrs. Weight 2,370 gm.
	241	16.8	60 min. after glucose. (10 gm. by stomach tube.)

in the beginning, that for some unknown reason, one or the other determination was entirely lost or had to be discarded because the duplicates were too far apart. On the whole, we would ascribe to the H_2SO_4 procedure, which was used in the majority of the blood determinations and exclusively for the analysis of lactic acid in tissues, an error of 7 to 10 per cent.

The normal values for the lactic acid content of the venous blood of rabbits and of cats, expressed in mg. per 100 cc. of blood, were as follows: normal rabbits, 13.1, 14.2, 14.8, 15.2, 16.2, 17.2, 21.5, 22.6, 23.3, 25.5, 27.6, 29.3, 31.8, 35.7, 39.7; phlorhizinized

rabbits, 32.7, 53.9; normal cats, 15.1, 15.4, 20.0, 20.7, 21.5, 22.0, 22.5, 22.8, 23.8, 23.8, 24.0, 24.1, 24.9, 27.2, 29.8, 34.1, 33.7; and depancreatized cats, 26.7, 34.7, 38.3.

The experiments on rabbits are recorded in Table I. Eight experiments were performed with insulin and in four cases insulin was followed by glucose in order to relieve hypoglycemic symptoms. In only two instances (Rabbits 1 and 2) was the lactic acid slightly increased after the administration of insulin, but a repetition of the experiments on the same rabbits failed to show this increase, even though the blood sugar dropped to the same low level. On three occasions (Rabbits 1 and 3) the blood was analyzed a few minutes after violent hypoglycemic convulsions and, as would be expected, the lactic acid was markedly increased. On the other hand, blood taken immediately before the onset of convulsions showed a normal lactic acid content, neither did a combination of insulin and glucose lead to an increase of the lactic acid. One experiment on a completely phlorhizinized rabbit (No. 4) was also negative. It would seem, therefore, that an increase in the lactic acid of the blood of insulinized rabbits is associated rather with excessive muscular movements than with a specific action of insulin. Three experiments were performed with epinephrine (Rabbits 5 and 6), and a strong increase in the lactic acid of the blood was noted. The lactic acid, however, did not parallel the blood sugar curve, for in two cases the highest lactic acid values occurred before the highest blood sugar was reached. In the third case (Rabbit 6) the experiment was performed on a poorly nourished animal, which did not show a very strong increase in the blood sugar following the administration of epinephrine, and the highest lactic acid value was observed when the blood sugar curve had already declined. A hyperglycemia produced by glucose did not cause a change of the lactic acid content of the blood (Rabbit 7).

The experiments on cats are summarized in Table II. In seven experiments with insulin (Cats 1 to 5) the same results were obtained as with rabbits. In two cases (Cats 2 and 3) there was an increase in the lactic acid after the insulin injection, but this could not be duplicated in a repetition of the experiments on the same animals. In one instance (Cat 3) blood was taken 5 minutes before convulsions supervened, when the blood sugar

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TABLE II.

Influence of Insulin and Epinephrine on the Lactic Acid Content of the Blood of Cats.

Cat. No.	Blood sugar.	Lactic acid.	Remarks.
	mg. per 100 cc.	mg. per 100 cc.	
1	116	22.5	Before insulin. Fasted for 24 hrs.
	74		55 min. after 4 units insulin.
	58	24.7	160 " " insulin. Symptoms of hypoglycemia. 5 gm. glucose subcutaneously.
	93	16.1	75 min. later.
2	99	22.8	Before insulin. Fasted for 24 hrs.
	100	15.1	" "
	61	36.7	180 min. after 2 units insulin.
	79	17.3	300 " " insulin.
2	84	23.8	Before insulin. Fasted for 24 hrs.
	57		60 min. after 2 units insulin.
	56		110 " " insulin.
	54	22.2	130 " " "
3	86	29.8	Before insulin. Fasted for 24 hrs.
	63	41.7	165 min. after 2 units insulin.
	100	32.1	300 " " insulin.
3	86	21.5	Before insulin. Fasted for 24 hrs.
	25	15.8	190 min. after 3 units insulin.
			195 " " insulin. Strong convulsions.
	211	22.9	5 gm. glucose subcutaneously. 60 min. later.
4	107	15.4	Before insulin. Fasted for 17 hrs.
	110	27.2	" "
	71	18.5	140 min. after 2 units insulin.
	81	22.7	230 " " insulin.
5	90	20.0	Before insulin. Fasted for 48 hrs.
	43	19.3	200 min. after 2 units insulin.
	45	27.5	290 " " insulin.
6	276	26.7	Before insulin. On the 3rd day after pancreatectomy. Fasted for 72 hrs.
	190	21.2	120 min. after 4 units insulin.

TABLE II—*Concluded.*

Cat No.	Blood sugar.	Lactic acid.	Remarks.
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
7	382	34.7	Before insulin. On the 4th day after pan- createctomy. Fasted for 80 hrs.
	346		85 min. after 8 units insulin.
	115	17.4	300 " " insulin.
7	350	38.3	Before insulin. On the 6th day after pan- createctomy.
	161		140 min. after 10 units insulin.
	45	19.3	320 " " insulin. Symptoms of hy- poglycemia.
8	84	24.1	Before epinephrine. Fasted for 24 hrs.
	222	41.4	95 min. after 0.3 mg. epinephrine.
	242	48.7	195 " " epinephrine.
8	99	20.7	Before epinephrine. Fasted for 24 hrs.
	99	24.0	" "
	186	34.7	200 min. after 0.1 mg. epinephrine.
	128	21.3	300 " " epinephrine.
8	94	23.8	Before epinephrine. Fasted for 24 hrs.
	440	31.1	130 min. after 0.7 mg. epinephrine.
	384	47.4	230 " " epinephrine.
	206	19.6	325 " " "
9	89	34.1	Before epinephrine. Fasted for 72 hrs.
	85	35.7	" "
	252	37.5	135 min. after 0.5 mg. epinephrine.
	303	30.6	240 " " epinephrine.
10	111	24.9	Before glucose. Fasted for 24 hrs.
	99	22.0	" "
	221	20.6	60 min. after glucose. (5 gm. by stomach tube.)
Dog 1.	107	18.4	Before insulin. Fasted for 48 hrs.
	74	19.0	180 min. after 8 units insulin.

was reduced to 25 mg., but the lactic acid content of the blood was normal. Glucose combined with insulin did not have an effect on the lactic acid content of the blood. In connection with other experiments on depancreatized cats a few observations as to the effect of insulin on the lactic acid of the blood could be made. In three cases (Cats 6 and 7) the lactic acid content was rather decreased after the insulin injection. It should also be noted that these completely depancreatized animals had a normal lactic acid content of the blood. Epinephrine was injected in four cases (Cats 8 and 9) and resulted in an increase in the lactic acid content of the blood, but the response was not so marked nor as constant as with rabbits. A hyperglycemia produced by other means, namely by the administration of glucose, failed to show an effect on the lactic acid content of the blood (Cat 10). One experiment on a dog did not show a change in the blood lactic acid following insulin.

2. Lactic Acid in Tissues.

Experiments on the influence of insulin and epinephrine on the lactic acid content of liver and of muscle were made on mice. A brief reference to the method of analysis used has already been made on another occasion (5). The departure from previous methods consisted in the removal of interfering substances by the Salkowski-Van Slyke (6) CuSO_4 and $\text{Ca}(\text{OH})_2$ precipitation, leaving the lactic acid in the filtrate, instead of extracting it with ether or amyl alcohol, which is a rather tedious procedure. Clausen (4) thought that the chief interfering substance in blood filtrates was sugar and he was the first to use the Salkowski-Van Slyke precipitation in conjunction with the lactic acid determination in blood. It occurred to the author that the Salkowski-Van Slyke sugar precipitation might equally well be used for the lactic acid determination in tissues, and a few preliminary experiments showed this to be the case. The principle embodied in the precipitation mentioned above has since been adopted by Meyerhof (7) and quite recently Hirsch-Kauffmann (8) from Embden's Laboratory gave a detailed record of the different steps involved in this method, so that a publication of our preliminary experiments may be omitted.

Our experiments were carried out as follows: the mice were

fasted for different lengths of time prior to the injections. The insulin was administered intraperitoneally in amounts of 0.1 to 0.2 cc., while the epinephrine was given subcutaneously. In producing insulin convulsions, use was made of the observation of Krogh (9), that mice kept at a temperature of 28–30°C. are more liable to get convulsions. Control mice were kept under the same conditions as the injected mice and one control mouse was always killed as simultaneously as possible with one injected mouse. The mice were stunned by a blow on the head, as much of the liver as could be quickly obtained was removed, and the liver placed on a watch-glass, covered previously with CO₂ snow. The liver was then immediately frozen solid in a stream of compressed CO₂, delivered from a tank. After weighing in the frozen state to the nearest mg., the liver was transferred to a small porcelain mortar which was cooled previously and which contained ice-cold 2 per cent HCl. The liver was crushed with a pestle and then ground to a very fine emulsion. The contents of the mortar were transferred quantitatively, with the aid of a brush, into a 10 cc. volumetric flask, and the volume made up to the mark with 5 per cent HgCl₂, about 5 cc. being used. After standing 1 to 3 hours, the proteins were filtered off. With muscle, which could not be ground up to such a fine state, it was found advantageous to let it stand overnight before filtration was started, but control experiments showed that 1 to 3 hours standing were sufficient for the liver to produce an even distribution of the lactic acid between precipitate and supernatant fluid. The filtrate from the proteins was freed from Hg and H₂S in the usual way, and 5 cc. of the final filtrate were carefully neutralized to litmus paper and made up to 10 cc. The precipitation of the interfering substances was then effected by adding 2 cc. of 10 per cent CuSO₄ solution and 2 cc. of a 5 per cent suspension of Ca(OH)₂. After standing for half an hour with occasional shaking, the precipitate was centrifuged down and two 5 cc. portions were used for the determinations of the lactic acid by Clausen's H₂SO₄ method. The von Fürth-Charnass (10) procedure, which is somewhat more accurate, could not be used, because the amounts of lactic acid present were too small. The muscles of the mice were worked up in exactly the same way. After stunning the animal, the skin around the hind legs, especially

along the line along which the legs had to be severed from the body, was frozen for a few seconds in a strong stream of compressed CO_2 . The legs could generally be severed from the body before suffocation convulsions of the animals set in. The preliminary freezing prevented twitching of the muscles, which otherwise occurs, when muscle is cut. Both legs were quickly skinned and immediately frozen, the work being divided between two persons. After weighing and crushing under ice-cold HCl , the muscles were carefully removed from the bones, and the bones weighed back in order to ascertain the amount of muscle taken.

The values recorded in the literature for the lactic acid content of resting mammalian muscle are considerably higher than those of cold blooded animals. Meyerhof (11) reported 0.06 to 0.16 per cent for rat muscles, Embden, Schmitz, and Meineke (12) 0.057 to 0.079 per cent for dog and 0.136 to 0.225 per cent for rabbit muscle, as compared with the standard value for resting frog muscle of 0.02 per cent. Apparently the time that elapses between the death of the animal and the checking of glycolysis is even under optimal conditions too long to prevent entirely an increase in the lactic acid content of the muscles of the warm blooded animals. Asphyxia undoubtedly plays the largest part in producing this increase in lactic acid. The time that elapsed in our experiments between the stunning of the mice and the freezing of either liver or muscle varied between 40 to 60 seconds, and our values agree well with those of other workers.

It was found that the length of time of fasting prior to the experiment had a certain influence on the lactic acid and free sugar content of the liver. The lactic acid and free sugar values for the liver of mice fasted for a short period were higher than for mice fasted for a long time, as will be seen from the data recorded in Table III. On the other hand, no influence of fasting on the lactic acid content of the muscles could be detected.

Tables IV and V summarize the data that were obtained on the influence of insulin, of insulin convulsions, and of epinephrine on the lactic acid content of the liver and muscles of mice. Each series consisted of an equal number of control mice and injected mice, which were killed as simultaneously as possible. In the series in which convulsions were produced by insulin, the mice were killed 30 to 60 seconds after a convulsive seizure. It should

be noted, however, that only part of these mice had strong convulsions, while others were in a state of coma which was interrupted by less violent convulsions.

TABLE III.

Influence of Fasting on the Lactic Acid and Free Sugar Content of the Liver.

Length of time of fasting.	Lactic acid in liver.	Free sugar in liver.	Remarks.
hrs.	gm. per 100 gm.	gm. per 100 gm.	
0-2	0.051	0.357	Average of 15 mice.
17-22	0.011	0.198	" " 10 "

TABLE IV.

Influence of Insulin, of Insulin Convulsions, and of Epinephrine on the Lactic Acid Content of the Liver of Mice.

Free sugar in liver.	Lactic acid in liver.	Remarks.
gm. per 100 gm.	gm. per 100 gm.	
0.255	0.020	Average of 12 control mice.*
0.171	0.016	" " 12 insulin " (no convulsions).*
0.267	0.031	" " 8 control mice.†
0.169	0.033	" " 8 insulin " (convulsions or coma plus convulsions).†
0.371	0.061	Average of 7 control mice.‡
0.554	0.057	" " 7 epinephrine mice.‡

* Mice fasted previous to the experiment from 2 to 22 hours. Average fasting time 12 hours. Insulin was injected intraperitoneally in doses of 0.04 to 0.08 units. The mice were killed from 40 to 80 minutes after the injection.

† Mice fasted previous to the experiment from 11 to 18 hours. Average fasting time 8 hours. The insulin was injected intraperitoneally in doses of 0.08 to 0.16 units. The time that insulin was allowed to act varied from 15 to 80 minutes. The mice were killed 30 to 60 seconds after a convulsive seizure.

‡ Mice not fasted prior to the experiment. Epinephrine was injected subcutaneously in doses of 0.05 to 0.1 mg. The mice were killed 15 to 40 minutes after the injection.

Since bleeding had to be avoided, blood sugar determinations could not be made. A substitute for the blood sugar and a reliable indication of the strength of the insulin and epinephrine actions was found in the determination of the free liver sugar,

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which was carried out in the same filtrate that was used for the lactic acid determination, using the Hagedorn and Jensen method. It was found previously (5) that the lowering of the free liver sugar is as constant a phenomenon of insulin action as is the lowering of the blood sugar. This observation is confirmed by the experiments in Table IV and extended, in that our previous data did not include insulin convulsions. It is noteworthy that even

TABLE V.

Influence of Insulin, of Insulin Convulsions, and of Epinephrine on the Lactic Acid Content of the Muscles of Mice.

Free sugar in muscle.	Lactic acid in muscle.	Remarks.
gm. per 100 gm.	gm. per 100 gm.	
0.078	0.099	Average of 6 control mice.*
0.079	0.083	" " 6 insulin " (no convulsions).*
	0.120	" " 5 control " †
	0.101	" " 5 insulin " (convulsions or coma plus convulsions).†
	0.117	Average of 5 control mice.†
	0.115	" " 5 epinephrine mice.†

* Mice fasted previous to the experiment from 1 to 19 hours. Average fasting time 6 hours. Insulin was injected intraperitoneally in doses from 0.08 to 0.16 units. The mice were killed from 30 to 90 minutes after the injection.

† Mice fasted previous to the experiment from 4 to 28 hours. Average fasting time 19 hours. The insulin dose was 0.16 unit. The time that insulin was allowed to act varied from 30 to 65 minutes. The mice were killed 30 to 60 seconds after a convulsive seizure.

‡ Mice fasted for 5 hours previous to the experiment. Epinephrine was injected subcutaneously in doses of 0.05 to 0.1 mg. The mice were killed from 20 to 45 minutes after the injection.

insulin convulsions do not raise the free liver sugar from its low level. In contrast to the free liver sugar, the free muscle sugar remains uninfluenced by insulin (Table V), which is in harmony with previous observations (5). Epinephrine was found by Palmer (13) to increase the free liver sugar of dogs, which was confirmed by Cori, Cori, and Pucher (14) for rabbits and guinea pigs. The present experiments show that epinephrine has the same effect on the free liver sugar of mice.

The lactic acid values in Tables IV and V do not show any decided influence of insulin or epinephrine on the lactic acid content of either liver or muscle. It was hoped to correlate the changes in the lactic acid content of the blood with similar changes in the lactic acid concentration in the tissues, and thus to find the source for the lactic acid increase in the blood that was observed after insulin convulsions and after epinephrine. This was however, not possible. Apparently, the lactic acid concentration in the tissues of the living animal is altered only under extreme conditions. Up to a certain limit an excess of lactic acid present in the tissues, which cannot be immediately removed by oxidation, coupled with reconversion into the precursor, seems to be disposed of by passing it into the blood stream. Otherwise it could not be explained why insulin convulsions produced an increase of the lactic acid of the blood, but failed to show a corresponding rise of lactic acid in the muscles.

It should be mentioned that the absence of an increase of lactic acid in the tissues following insulin is not due to a lack of glycogen. The behavior of the liver glycogen during insulin action has been studied on a former occasion (15), and there is no doubt that there is enough glycogen present in the liver even during insulin convulsions. As regards the muscle, Dudley and Marrian (16) reported one experiment on a rabbit, which was killed at the onset of convulsions, 6 hours after the insulin injection, and which showed a complete absence of muscle glycogen. This observation could not be confirmed on mice. The combined muscles of four control mice, fasted for 5 hours previously, showed a muscle glycogen of 0.15 per cent, while the combined muscles of four mice, which were fasted for the same length of time and which were killed after insulin convulsions, showed 0.120 per cent of muscle glycogen. A similar experiment in which the mice were not fasted previously and in which the injected mice were killed before they had convulsions gave 0.182 per cent of muscle glycogen as an average of five control mice and 0.150 per cent of muscle glycogen as an average of five insulin mice. In view of these data the behavior of the muscle glycogen during insulin action seems to need a revision. That there was abundant carbohydrate present in the muscles was also revealed by the fact that chloroform or caffeine rigidity produced on the muscles

of insulinized mice, fasted for 24 hours previously, gave values up to 0.25 per cent of lactic acid.

There is only one reference in the literature where the lactic acid in tissues following insulin was determined under conditions similar to ours. Collazo, Händel, and Rubino (17) state, without giving protocols, that in a number of guinea pigs, which were fasted previously for 14 hours and then received, 4 hours before death, 3 gm. of glucose and 7 units of insulin, the lactic acid content of the muscles was the same or slightly lower than in suitable control animals. In regard to epinephrine there is a report on lactic acid determinations in the liver by Elias and Sammartino (18). Five control rabbits showed as an average 0.038 per cent of lactic acid, while seven rabbits, treated with epinephrine and killed from 1 to 3 hours after the injection, contained as an average 0.100 per cent of lactic acid. Later Sammartino (19) found 0.051 per cent of lactic acid as an average of six experiments on rabbits on which the piqure had been performed. Our experiments recorded in Table IV do not show an increase in the lactic acid content of the mouse liver after epinephrine.

SUMMARY.

Taking the results as a whole, neither the analysis of the lactic acid content of the blood nor of the tissues favors the hypothesis that insulin influences the reaction $\text{glucose} \rightleftharpoons \text{lactic acid}$ in the direction to the right, as was postulated by Briggs, Koechig, Doisy, and Weber (1). In fact, the later work of these authors (20) seems to contradict the hypothesis, since they found that completely depancreatized dogs show a strong increase in the lactic acid of the blood and tissues, when subjected to strychnine convulsions. Tolstoi, Loebel, Levine, and Richardson (2), studying the effect of insulin on the lactic acid content of the blood of diabetic subjects, found that the fall in blood sugar following insulin administration was not always accompanied by a rise in lactic acid, nor did they find a correspondence between the fall in blood sugar and an increase in the lactic acid. Furthermore, one patient, on whom three separate observations were made, showed only in one instance an increase in lactic acid of the blood after insulin. The effect of epinephrine was studied

by these authors on normal persons. There was in all cases a decided rise in the lactic acid of the blood 1 hour following the epinephrine injection. Tolstoi and associates believe that the factor common to the action both of insulin and epinephrine, which increases the lactic acid in spite of the divergent effects on the blood sugar, might be a local asphyxia. If this conception is correct it would mean that the increase in the lactic acid of the blood following insulin or epinephrine is not specific in nature.

CONCLUSIONS.

1. Insulin hypoglycemia produces no definite change in the lactic acid content of the blood of either fasting rabbits or cats, nor does insulin have an effect on the blood lactic acid of phlorhizinized rabbits or depancreatized cats.

2. Insulin convulsions lead to a strong increase in the lactic acid concentration of the blood.

3. Epinephrine causes a rise in the blood lactic acid of rabbits and cats. The effect is more marked in the former animals than in the latter.

4. The lactic acid concentration in the liver and in the muscles of mice remained uninfluenced by insulin hypoglycemia, by insulin convulsions or coma plus convulsions, and by epinephrine. The free sugar content of the liver was strongly lowered by insulin and remained low in spite of insulin convulsions.

5. It is concluded that insulin has no effect on the reaction $\text{glucose} \rightleftharpoons \text{lactic acid}$ in the direction to the right.

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THE FORMS OF NITROGEN FOUND IN CERTAIN LAKE WATERS.*

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Previous investigations (1) carried on by the Wisconsin Geological and Natural History Survey have shown that the water of Lake Mendota contains more than nine times as much soluble nitrogen as total plankton nitrogen. This fact led to the present study of the different forms of soluble nitrogen and soluble organic matter in Wisconsin lake waters, with a more detailed study on the Mendota waters.

In the case of Lake Mendota a drainage basin of about 250 square miles discharges its water into this lake (2). A part of this water enters as surface drainage by means of the Yahara River, Five Mile Creek, and Pheasant Branch Creek. Another portion is derived from the numerous springs which are located chiefly at the west end of the lake.

The various forms of nitrogen in the lake water serve either directly or indirectly as the source of nitrogen for both the plant and animal life of the lake. Most land plants utilize nitrates and ammonia salts as their chief supply of nitrogen, and it is generally accepted that water plants likewise utilize nitrates. Rice (3) has recently pointed out that the growth of large plants in Lake Winona is accompanied by a reduction in the nitrate content of the water. The aquatic animals obtain their nitrogen chiefly through feeding upon chlorophyll-bearing plants (4). Pütter (5), however, has advanced the view that aquatic animals may derive their nutrients solely from organic compounds dissolved in the

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water. This theory is not generally accepted and has been opposed by several investigators, particularly Lipschütz (6) and Lantzsch (7).

The origin of the soluble nitrogenous compounds may be attributed to the inflowing water, the decomposition of the insoluble organic material, and the excretions of aquatic animals.

Many factors such as temperature, food supply, and stratification of the water operate to bring about a succession of plankton and bacterial forms during the year. A similar change in the forms of nitrogen is to be expected and in the experimental part of this paper data will be presented to show how these forms vary.

EXPERIMENTAL.

Samples.—In carrying out this investigation, 77 samples of water varying in size from 50 to 525 liters have been collected and analyzed besides a large number of 1 and 2 gallon samples. In all about 7,000 liters of water were obtained from ten different lakes. The source, number, and size of the samples are as follows:

Mendota surface water (50 to 500 liter samples).....	32
“ bottom “ (50 “ 300 “ “).....	19
Waters flowing into Mendota (50 to 100 liter samples).....	5
“ other than Mendota (50 to 200 liter samples).....	21
<hr/>	
Total.....	77

The collection and analysis of such large quantities of water have not, so far as we know, been attempted in previous investigations and have made possible a study of forms of nitrogen which could not be determined on small samples of water. Most of the samples were taken from the surface or bottom of Lake Mendota, as it was desired to have a continuous record over the entire 2 years of work in order to determine any seasonal changes in the forms of nitrogen. The bottom samples were taken by means of a pump and hose at a depth of 20 meters where the total depth is 23 meters. The water was centrifuged immediately upon arriving at the laboratory by means of a Sharples centrifuge at a speed of about 40,000 R.P.M. The flow of water was so regulated that about 1 liter passed through the machine every 3 minutes. All the plankton and about 70 per cent of the bacteria

were removed by this procedure. The sediment which gathered in the centrifuge bowl was washed into a tared platinum dish, dried at 65°C., weighed, and the nitrogen determined by the Kjeldahl method. In the tables it is reported as plankton nitrogen. In the early part of this work the centrifuged water was poured into large porcelain dishes and evaporated on a steamer at a temperature below 70°C. with the aid of electric fans, to a small volume, 250 or 500 cc. Beginning October 5, 1922, the samples were evaporated in a copper vacuum pan by which means large quantities of water could be concentrated to a small volume within a few hours. During the evaporation the vacuum was held around 25 inches of mercury and the temperature at about 60°C. In order to test the two methods of evaporation, two samples were evaporated simultaneously, one according to the old method and the other by means of the vacuum pan, but analyses indicated no difference in the forms of nitrogen present.

Methods of Analysis.

Plankton and Soluble Nitrogen.—Plankton and soluble nitrogen were determined by the Kjeldahl method. In the case of soluble nitrogen, duplicate aliquots were generally taken, and were equivalent to 10 liters of the original water. With samples containing nitrates, the Jodlbauer (8) modified Kjeldahl method was used. The total soluble nitrogen of samples whose free ammonia nitrogen was also determined is reported in the tables as the sum of both the free ammonia and the Kjeldahl nitrogen. In all cases the results are given on the basis of milligrams of nitrogen per cubic meter (1,000 liters) of water.

Ammonia Nitrogen.—The free ammonia nitrogen in the original water was determined according to the method given by the American Public Health Association (9). The ammonia that remained in the sample after evaporation, called residual ammonia, was determined by Folin's method (10).

Nitrite Nitrogen and Nitrate Nitrogen.—These two forms of nitrogen were determined on the original water immediately upon arriving at the laboratory. The procedure as given by the American Public Health Association was followed (11).

Amino and Non-Amino Nitrogen.—The amino nitrogen in each sample was determined by Van Slyke's method (12), using a

special gas burette to take care of large amounts of gas evolved. This burette combined the graduated tube of the micro burette with the large bulb of the macro apparatus. The free amino nitrogen was determined upon 10 cc. aliquots of the unhydrolyzed, evaporated residues, equivalent to 2 liters of the original water. The total amino nitrogen was determined upon 10 cc. aliquots of the evaporated water which had been hydrolyzed for 6 hours with 20 per cent HCl under a reflux condenser. A number of experiments showed that hydrolysis for 6 hours yielded the maximum total amino nitrogen. The peptide nitrogen was obtained by difference between these two determinations. The amino nitrogen was determined in some cases not only by the Van Slyke method but also by the Folin (13) and Sörenson (14) methods.

The non-amino nitrogen was obtained by subtracting the sum of the two forms of ammonia nitrogen, the nitrite, the nitrate, and the total amino nitrogen from the total soluble nitrogen. In cases where only the free ammonia nitrogen was determined an average of 20 mg. was taken as the residual ammonia nitrogen.

Protein, Peptone, and Diamino Acid Nitrogen.—The more complex forms of nitrogen, such as proteins, peptones, and diamino acids, were determined by precipitation with phosphotungstic acid as follows: a 50 cc. aliquot of the concentrated water, equivalent to 10 liters of the original water, was treated with the phosphotungstic acid reagent and the volume made up to 100 cc. The precipitate was filtered off and 75 cc. of the filtrate were used for the determination of total nitrogen by the Kjeldahl method. The amount of nitrogen precipitated was obtained by difference and is designated as "N precipitated by phosphotungstic acid" in Tables I and II.

*Nature and Seasonal Variation of Different Forms of Nitrogen
Occurring in Mendota Water.*

The nitrogen content of the water of Lake Mendota was separated into different fractions by the methods just described. The various forms of nitrogen are closely related to one another and may display changes, depending upon the nature of this relation, which are similar or opposite in direction. The variations are dependent upon such factors as stratification of the

TABLE I.
Forms of Nitrogen in Surface Water of Lake Mendota.
Station II.

Date.	Plankton N.	Soluble N.*	N precipitated by phosphotungstic acid.	Ammonia N.†	Nitrite N.	Nitrate N.	Free amino N.	Peptide N.	Non-amino N.
	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.
1921									
Sept. 28	151.0	467.0		20.0			37.0	227.0	183.0
1922									
Feb. 8	159.0	969.0		12.0		8.0	236.0	436.0	277.0
June 7	154.0	586.0	216.0	16.0			140.0	223.0	207.0
" 27	102.3	625.0	336.5	11.6			82.0	253.1	278.3
July 17	179.4	377.1	121.2	13.3		16.5	81.2	88.5	177.6
Sept. 15	66.2	378.0	122.6	23.5		11.1	73.2	77.0	193.2
Oct. 5	89.9	453.5	132.5	39.2		13.9	108.6	135.2	156.6
Nov. 2	90.7	483.8	114.2	20.2	10.9	29.6	110.0	121.0	192.1
" 21	114.4	514.1	144.5	20.1	9.2	29.6	124.9	173.7	156.5
Dec. 5	144.1	538.2	162.0	24.2	14.3	16.1	135.8	179.2	168.6
" 19	63.5	554.2	191.6	30.2	15.4	14.2	146.8	213.3	134.3
1923									
Jan. 9	40.4	566.6	195.3	20.2	17.9	21.8	111.1	241.1	154.5
Feb. 1	39.4	597.1	220.1	30.3	10.6	24.0	105.4	255.3	171.5
" 28	51.6	914.4	225.4	260.0	10.0	90.4	101.6	249.9	202.5
Mar. 28	73.1	660.2	202.8	132.0	5.2	84.8	80.1	181.5	176.6
Apr. 25	175.5	939.7	216.7	168.0	3.8	229.9	81.7	234.1	222.2
May 18	83.8	937.3	225.1	150.4	4.0	214.3	89.8	234.2	244.6
June 8	242.0	786.2	197.2	116.0	6.2	122.5	67.9	222.6	251.0
July 2	99.6	645.5	201.5	92.0	3.0	48.1	62.4	203.7	236.3
" 24	112.8	527.7	140.9	96.0	1.8	23.2	63.4	121.4	221.9
Aug. 1	82.7	546.9	135.4	100.0	1.6	25.0	58.0	129.5	232.8
" 22	110.0	529.8	127.5	88.0	1.7	17.9	53.1	130.1	239.0
Sept. 21	91.3	478.4	113.0	98.4	0.0	8.3	48.1	125.4	198.4
Oct. 24	45.3	442.6	124.6	124.0	8.9	20.5	35.5	119.9	133.8
1924									
Jan. 7	77.8	484.2	124.9	140.0	12.5	25.0	55.0	118.3	133.4
Mar. 12	104.6	555.0	142.0	180.0	11.1	72.6	64.4	131.4	95.5
Apr. 23	79.8	574.4	150.4	144.0	12.2	83.3	73.0	137.0	124.9
May 26	82.9	557.0	152.0	132.0	11.0	79.2	77.9	139.1	117.8
June 18	92.4	515.6	146.7	116.0	10.0	69.4	54.0	135.0	131.2

* Residual ammonia included from Sept. 28, 1921 to Feb. 1, 1923; free and residual ammonia Feb. 28, 1923 to June 18, 1924.

† Residual ammonia Sept. 28, 1921 to Feb. 1, 1923; free and residual ammonia, Feb. 28, 1923 to June 18, 1924.

Nitrogen in Lake Water

TABLE II.
Forms of Nitrogen in Bottom Water of Lake Mendota.
Station II.

Date.	Plankton N.	Soluble N.*	N precipitated by phosphotungstic acid.	Ammonia N.†	Nitrite N.	Nitrate N.	Free amino N.	Peptide N.	Non-amino N.
	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.
1922									
July 3	61.6	476.7	153.5	7.3		55.3	86.4	144.0	183.7
" 21	49.5	422.7	135.3	16.7	45.2	46.4	99.3	117.3	97.8
Sept. 26	46.6	380.4	89.2	26.9	21.7	27.8	109.7	128.0	66.3
Oct. 31	187.5	504.0	121.0	20.2	9.1	32.0	110.0	141.3	191.4
Dec. 5	144.1	538.2	162.0	24.2	14.3	16.1	135.8	179.2	168.6
1923									
Jan. 9	103.2	607.1	188.9	30.3	16.1	35.7	108.0	244.0	173.0
Feb. 1	67.2	738.6	319.6	40.4	25.0	40.4	117.0	266.0	249.5
" 28	56.8	1,739.2	319.2	756.0	57.7	366.3	109.8	263.7	185.7
Mar. 28	47.2	1,505.2	240.2	764.0	36.4	227.3	85.4	197.6	194.5
Apr. 25	175.5	939.7	216.7	168.0	3.8	229.9	81.7	234.1	222.2
May 18	83.8	937.3	225.1	150.4	4.0	214.3	89.8	234.2	244.6
June 8	90.0	1,148.7	208.7	372.0	5.8	204.5	92.3	236.2	237.9
July 2	65.8	1,212.3	242.1	468.0	4.2	127.5	97.7	238.9	276.0
" 24	58.2	936.0	130.0	420.0	40.3	71.5	71.3	121.4	211.5
Aug. 22	64.0	962.7	137.7	460.0	27.7	46.3	69.0	122.1	237.6
Sept. 21	98.5	958.9	130.0	548.0	10.5	24.5	53.4	117.4	205.1
Oct. 24	46.3	442.6	124.6	124.0	8.9	20.5	35.5	119.9	133.8
1924									
Jan. 7	77.8	484.2	124.9	140.0	12.5	25.0	55.0	118.3	133.4
Mar. 12	127.7	1,405.2	137.0	748.0	60.0	227.2	96.6	139.5	133.9
Apr. 23	79.8	574.4	150.4	144.0	12.2	83.3	73.0	137.0	124.9
June 25	44.9	766.9	162.9	300.0	17.0	92.6	81.0	140.4	135.9

* Residual ammonia included from July 3, 1922 to Feb. 1, 1923; free and residual ammonia Feb. 28, 1923 to June 25, 1924.

† Residual ammonia July 3, 1922 to Feb. 1, 1923; free ammonia and residual ammonia Feb. 28, 1923 to June 25, 1924.

water, temperature, light, oxygen supply, and the effect of in-flowing and outflowing water. The most sudden changes are due to a mixing of the stratified water when the spring and autumn "turnovers" occur. At this time the forms and quantity of nitrogen are the same throughout the entire lake. The samples

taken at such times are designated in the tables, to show the occurrence of a turnover. The data for the different forms of nitrogen are given in Tables I and II and Charts 1 and 2 and will be discussed in the order there listed.

Plankton Nitrogen.—This form of nitrogen consists of the plants and animals that inhabit the fresh waters and range from forms as simple as bacteria and algæ to those as complex as crustacea

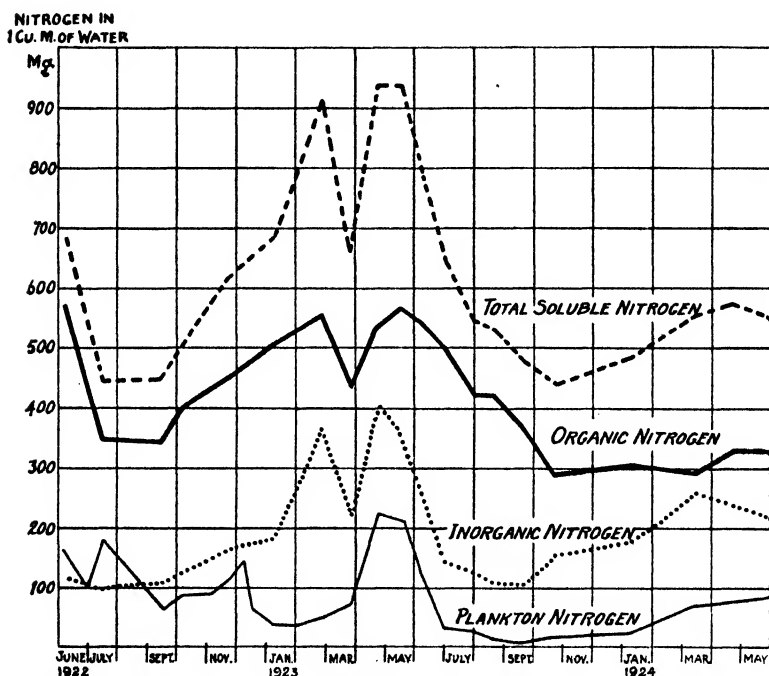


CHART 1. Forms of nitrogen in the surface water of Lake Mendota.

and insect larvæ. The plankton obtained by centrifuging the water was dried and weighed, and the nitrogen content determined. This was found to average about 3.5 per cent of the dry material. There is at all times a great excess of soluble nitrogen over that found as insoluble or plankton nitrogen; the former is from three to twenty times as large as the latter.

The plankton nitrogen in both top and bottom waters shows seasonal variations. A maximum is reached in spring and is

then followed by a decline in midsummer. A second rise takes place in fall and is succeeded by another low level in late fall or winter. As the phytoplankton feed upon the soluble nitrogen, the latter decreases as the former increases. A comparison of the surface and bottom waters shows the plankton nitrogen to be higher in the surface than in the bottom during the summer, while in the winter the bottom exceeds the surface in its content of plankton.

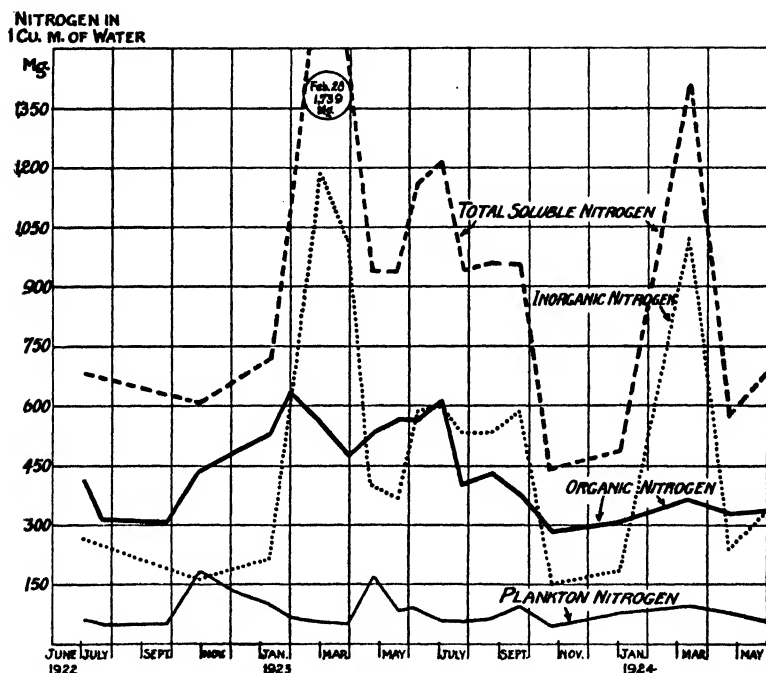


CHART 2. Forms of nitrogen in the bottom water of Lake Mendota.

Soluble Nitrogen.—The nitrogen in this form is the source of supply for the plankton bacteria, and other forms of life, and is from three to twenty times as large in amount as that existing in the insoluble state. It results from the decomposition of organic material by bacteria and other microorganisms and consists of a number of chemical compounds such as proteins, amino acids, ammonia, nitrates, etc. Most of the soluble nitrogen is

formed at the bottom of the lake from whence it spreads upward toward the surface. The marked changes appear first, and are more pronounced in the bottom water than in the surface water. At the spring and fall turnovers, the soluble nitrogen content of the lake is uniform. As soon as stratification sets in, the nitrogen in the bottom layer exceeds that in the surface zone. The maximum in both waters occurs in late winter, and the minimum toward the end of the summer. The causes for high and low levels will be considered more fully in connection with ammonia and nitrates.

TABLE III.

Precipitation of the Organic Nitrogen Contained in Lake Mendota Water by Various Reagents.

Sample June 3, 1922.

Reagent.	Reagent* in 100 cc.	Total nitrogen.	Nitrogen not precipitated.	Nitrogen precipitated.	
				mg. per cu. m.	per cent
Phosphotungstic acid.....	8.0	558.0	351.0	207.0	37.1
Mercuric chloride.....	8.0	558.0	400.0	158.0	28.3
Tannic acid.....	2.0	558.0	416.0	142.0	25.5
Lead subacetate.....	2.8	558.0	422.5	135.5	24.3
Sodium tungstate.....	1.0	558.0	430.9	127.1	22.8
Potassium mercuric iodide.....	3.4	558.0	440.0	118.0	21.2
Mercuric sulfate.....	2.0	558.0	448.0	110.0	19.7
“ chloride + 2 cc. concentrated HCl.....	8.0	558.0	400.0	158.0	28.3
Trichloroacetic acid.....	2.0	558.0	488.0	7.0	12.6

* 50 cc. samples; reagent and water to 100 cc.

Forms of Organic Nitrogen.—An attempt was made to separate the organic nitrogen into various fractions by means of several protein precipitants. The precipitants and weight used are given in Table III and are listed in descending order according to the quantity of nitrogen precipitated. The most efficient was phosphotungstic acid and the least efficient was trichloroacetic acid. Intermediate between these two and approximately of equal value were lead acetate, tungstic acid, tannic acid, and potassium mercuric iodide. According to Hiller and Van Slyke (15), tungstic acid precipitates not only the native proteins but also

the intermediate products, while trichloroacetic acid leaves the the derived proteins in solution. Phosphotungstic acid has long been used as a precipitant for proteins, proteoses, peptones, and diamino acids. Tannic acid is known to precipitate native proteins, intermediate products, and peptones. According to this basis the distribution of the precipitable nitrogen in the June sample is approximately as follows: proteins 12.6 per cent; intermediate products 10 per cent; diamino acids 15.3 per cent; and monoamino acids by difference 60 per cent. Owing to the small amount of nitrogen involved and the incompleteness of precipitation the above figures can be regarded as only approximately correct.

An attempt was made to increase the amount of nitrogen precipitable with phosphotungstic acid by concentrating the aliquot and by increasing the quantity of reagent, but no more nitrogen was obtained in the precipitate. Since phosphotungstic acid made the best division between precipitable and non-precipitable nitrogen, it was adopted as the reagent in all of the analyses given in Tables I and II.

Ammonia, Nitrites, and Nitrates.—These compounds comprise from 25 to 50 per cent of the total soluble nitrogen. They originate in all probability from the decomposition of organic forms of nitrogen contained in the mud and debris at the bottom of the lake. They are in general more abundant in the bottom than in the surface waters. Sudden changes in their concentration also appear first and are more pronounced in the lower levels. An understanding of these changes can be best understood by considering the variations which occur in the bottom water.

After the fall turnover there is very little change until some time in February when there is a sudden and enormous increase in ammonia, nitrites, and nitrates. From February 1 to 28, 1923, the nitrates increased more than 900 per cent. In the following year from January 7 to March 12, all three forms of nitrogen made increases amounting from 500 to 900 per cent. Analyses made between these dates show that a 200 per cent increase may take place in 1 week. The increases are observed first in the bottom water and later at various depths until finally they can be detected at the surface. As yet it is not clear what causes are responsible for the large and sudden increases, but it is probable that tem-

perature, oxygen, light, and ammonia- and nitrate-producing bacteria are concerned. A special study of these factors is now being made and will be reported in a later paper.

In spring when the surface and bottom waters become thoroughly mixed, the lake becomes uniform throughout and the bottom and surface samples contain the same quantity of ammonia and nitrates. When stratification again takes place in early summer, the nitrates decrease, and the ammonia increases in the lower stratum. At this time oxygen is practically absent from the lower waters and denitrifying organisms make their appearance.

The lowest concentration of ammonia, nitrites, and nitrates in the surface zone occurs in the late summer and early fall. The plant life is still using considerable quantities of these forms of nitrogen and but little reaches the upper levels from the bottom strata at this time of the year.

Amino and Non-Amino Nitrogen.—The free amino nitrogen varies from 5 to 15 per cent, and the peptide nitrogen from 15 to 35 per cent of the soluble nitrogen. Both increase slowly through the fall and winter, and then decrease in the spring and summer. Neither shows the violent changes noted for ammonia and nitrates. They are slightly higher in the bottom water than in the surface water, but no marked difference in their concentration manifests itself in the samples from these two levels. The data are represented graphically in Charts 3 and 4.

As a further check on the accuracy of these data, the amino nitrogen was determined in four samples by two other methods. The data are given in Table IV, and show good agreement among all three methods. The Van Slyke and Folin methods run close together, while the Sörensen method gives somewhat higher results than either of the other two. The presence of amino nitrogen has been further established by identifying definite amino acids by means of both qualitative and quantitative methods. Tryptophane, tyrosine, histidine, cystine, and arginine have been found in these waters. The data will be given in the following paper. All of these results together with those obtained by the protein precipitants clearly establish the existence in solution of proteins and their decomposition products in fresh water lakes.

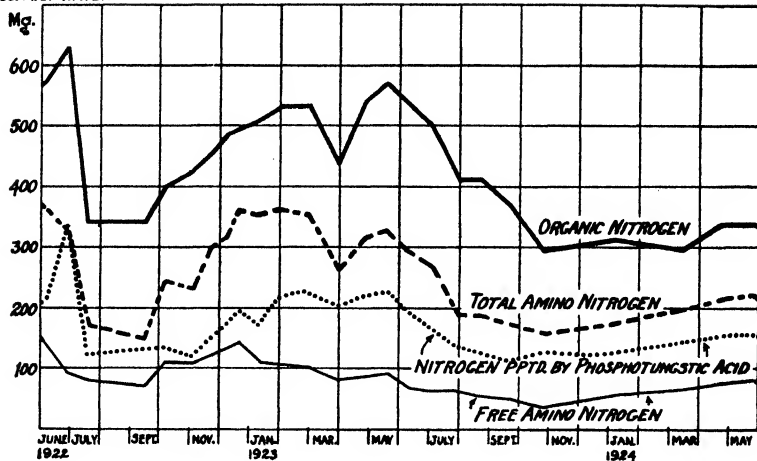
NITROGEN IN
1 CU. M. OF WATER

CHART 3. Forms of organic nitrogen in the surface water of Lake Mendota.

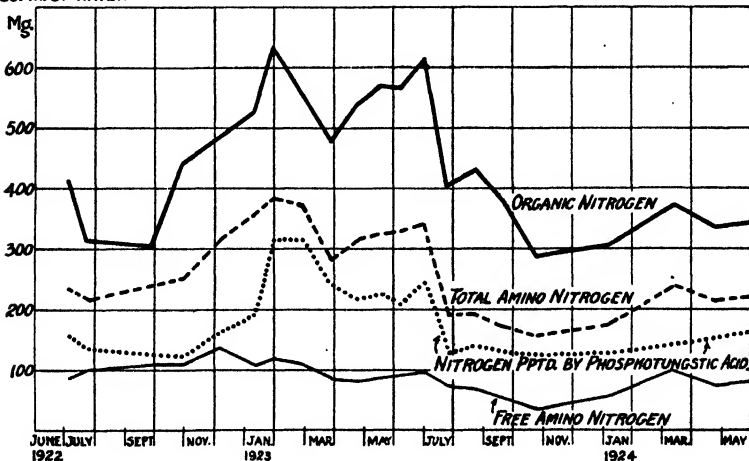
NITROGEN IN
1 CU. M. OF WATER

CHART 4. Forms of organic nitrogen in the bottom water of Lake Mendota.

The non-amino nitrogen, equivalent to from 20 to 40 per cent of the total soluble nitrogen, consists not only of the non-amino nitrogen of amino acids, but also includes small quantities of nitrogen in the form of amides, amines, and purines. In general it varies with the amino and peptide nitrogen, but shows a tendency toward a prolonged high level in summer, and a low level in winter. Since it is obtained by the difference between the total soluble nitrogen, and the sum of five other forms, the figures in the table show greater irregularities than for the other determinations.

TABLE IV.

Amino Nitrogen in the Lake Waters According to Different Methods of Analyses.

Sample.	Date.	Method.	Free NH ₂ -N.	Total NH ₂ -N.
	1922		mg. per cu. m.	mg. per cu. m.
Mendota surface.	June 7	Van Slyke.	12.31	266.30
	" 7	Sörenson.	14.00	333.00
	1923			
Devil's Lake.	Oct. 10	Van Slyke.	69.50	137.20
	" 10	Folin.	71.41	136.20
	" 10	Sörenson.	81.90	148.40
	1924			
Mendota surface.	Mar. 12	Van Slyke.	64.40	195.80
	" 12	Folin.	66.70	181.80
" bottom.	" 12	Van Slyke.	96.60	236.10
	" 12	Folin.	86.20	222.20

Equal values for the different forms of nitrogen are not obtained for 2 successive years because the conditions are not identical. Differences in rainfall, temperature, oxygen, etc., are reflected in the fauna and flora of the lake as well as in the nitrogen content. The general trend of the changes, however, is the same in succeeding years. It must be borne in mind that since there is a large inflow and outflow of water, the curves will show marked irregularities. General tendencies are far more significant than particular values.

Composition of Inflowing Water.—Forms of nitrogen in the water flowing into Lake Mendota were determined at different times of the year in order to get an idea of its composition and its probable

TABLE V.
Forms of Nitrogen in Waters Other than Lake Mendota.

Sample.	Date.	Plankton N. mg. per cu. m.	Soluble N. mg. per cu. m.	N precipitated by acid. mg. per cu. m.	Ammonia N. mg. per cu. m.	Nitrite N. mg. per cu. m.	Nitrate N. mg. per cu. m.	Free amino N. mg. per cu. m.	Peptide N. mg. per cu. m.	Non-amino N. mg. per cu. m.
Devil's Lake.....	1922 Oct. 27	38.3	337.7	136.1	15.1*	0.0	20.8	21.8	103.6	176.4
Wingra ".....	1923 June 6	882.0	896.0	298.0	104.0	0.0	40.0	78.9	299.5	343.6
Monona ".....	" 28	388.8	885.8	326.6	119.2	3.6	50.9	49.0	315.8	347.3
Rock ".....	July 6	73.1	683.0	143.0	100.0	1.8	31.3	120.5	176.7	252.7
Devil's ".....	" 11	15.2	281.4	132.4	68.0	0.0	0.0	74.9	72.4	71.3
Geneva ".....	" 16	50.9	457.7	135.3	92.0	1.0	27.5	74.5	135.9	126.8
Green ".....	" 18	42.3	424.0	130.3	84.0	1.7	28.8	93.3	96.0	120.5
Kegonsa ".....	" 20	696.6	839.4	379.0	88.0	0.0	21.1	99.7	299.3	331.3
Waubesa ".....	" 20	299.0	842.6	367.2	76.0	0.0	22.7	115.5	294.0	334.4
Wisconsin River.....	" 30	161.4	655.8	352.0	148.0	0.0	21.2	83.5	302.8	100.3
Devil's Lake.....	Oct. 5	14.3	314.3	110.4	124.0	0.0	11.7	69.5	67.7	39.3
Wisconsin River.....	" 12	90.3	506.4	143.8	116.0	0.0	19.3	54.1	143.5	173.5
Green Lake.....	" 17	48.7	460.8	136.8	132.0	2.0	30.4	70.9	122.7	102.8
Wisconsin River.....	Nov. 21	53.4	521.2	209.2	100.0	1.6	26.1	64.5	277.1	51.9
Madeline Lake.....	Dec. 12	95.6	616.5	150.5	144.0	2.5	15.4	82.2	151.1	221.3
Bass ".....	" 20	33.6	600.6	176.6	164.0	2.0	8.3	63.3	177.7	185.3

	1924											
Turtle Lake.....	Jan. 18	30.5	650.6	196.6	132.0	4.2	27.8	82.5	192.5	211.6		
Michigan ".....	Feb. 28	20.5	383.4	58.2	126.4	9.6	104.1	40.5	56.9	45.9		
Yahara River.	1923											
Before rain.....	July 6	609.3	596.2	301.2	14.7*	0.0	189.1	144.0	141.9	86.5		
After ".....	" 10	485.0	513.8	258.0	10.7*	0.0	78.7	107.1	160.1	137.2		
Warner's Spring.....	Aug. 2	0.0	2,081.0	43.0	5.0*	0.0	2,058.0	0.0	0.0	18.0		
	1923											
Yahara River.....	Mar. 5	0.0	4,892.0	26.0	0.0	0.0	4,888.0	0.0	0.0	4.0		
".....	" 14	186.1	1,967.1	107.1	260.0	4.6	1,076.7	74.7	186.7	364.4		
Wingra Spring.....	July 12				40.0	1.8	2,702.0					

* Does not include free ammonia.

effect on Mendota water. Some of the results are given in Table III. The water of Warner's Spring contained a larger amount of nitrates in winter than in summer. The Yahara River was analyzed before and after a heavy rainfall, and the results show a marked drop in the nitrogen content after the rain. In winter the Yahara River water showed an increase in nitrate nitrogen at a time when a rise in the nitrates occurred in Lake Mendota. While a considerable amount of nitrate nitrogen was entering Mendota through the river and the spring, yet this amount could account for only a small fraction of the increase in nitrates in the water during the winter months.

Nitrogen in Waters Other than Mendota.

The forms of nitrogen in waters of other lakes, Table V, show no marked variation from those of Mendota water taken about the same time of the year. The total soluble nitrogen in Devil's Lake, a soft water lake, is considerably lower than that found in Mendota. In contrast, the total nitrogen, amino nitrogen, and non-amino nitrogen in Wingra, Monona, Waubesa, and Kegonsa are considerably higher than those of Mendota. Lake Kegonsa and Lake Wingra are the only lakes of the list that had a large amount of plankton, the plankton nitrogen being about equal to the total soluble nitrogen. The Wisconsin River, whose source of water supply lies in the numerous northern Wisconsin lakes, contains about the same amount of the different forms of nitrogen as the northern lakes. In Lake Michigan all of the forms of nitrogen excepting nitrates are much lower than in any of the other lakes analyzed.

SUMMARY.

There is a seasonal variation in the different forms of nitrogen found in Lake Mendota, an inland lake of Wisconsin. This variation was noted in the surface water, in the bottom water, and in the inflowing water.

Ammonia, nitrites, nitrates, amino acids, and proteins reach a maximum in the winter, and fall to a minimum during the summer. A sudden and marked increase in ammonia and nitrates occurs in February. The bottom water always contains more of the different forms of soluble nitrogen than the surface water.

The plankton (plant material) nitrogen increases as the soluble nitrogen decreases, but shows marked spasmodic changes. These irregularities are due to the different crops of plankton which follow one another in rapid succession.

The forms of nitrogen in twelve other inland lakes of Wisconsin and in Lake Michigan are approximately the same as those found in Lake Mendota.

The seasonal variation in the different forms of soluble nitrogen indicates that these compounds form part of the nutrients of both plant and animal life of these waters.

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THE OCCURRENCE OF AMINO ACIDS AND OTHER ORGANIC NITROGEN COMPOUNDS IN LAKE WATER.*

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Although little is known regarding the nature of the soluble organic nitrogen in surface waters, there is no doubt but that it contains compounds of great biological significance to micro-organisms, plants, and animals. The superiority of organic forms of nitrogen as compared with inorganic compounds for the nutrition of the majority of bacteria and related micro-organisms is well known. Likewise in the growth of plants, water cultures containing amino acids, according to Schreiner and Skinner (1), gave better results than nutrient solutions containing only inorganic compounds of nitrogen. For a number of years, the reports of Pütter (2) have been the center of a controversy as to whether or not aquatic animals can utilize the organic nitrogenous compounds dissolved in the water. A knowledge of the nature of the organic nitrogenous compounds contained in surface waters would be of value in determining whether Pütter's theory is within the realm of possibility. Obviously, if the water does not contain such indispensable amino acids as tryptophane, cystine, tyrosine, and histidine, it cannot furnish the nutrients required by aquatic animals. In the preceding paper (3) it was shown that lake and river waters contain considerable quantities of organic nitrogen which can be precipitated by phosphotungstic acid and other protein precipitants. It was also found that this organic nitrogen contained amino, peptide,

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and non-amino nitrogen. In this paper, data will be presented to establish the existence of amino acids, purines, and other forms of organic nitrogen in such waters.

EXPERIMENTAL.

Lakes Studied.—Four inland lakes of Wisconsin and Lake Michigan have been included in this study. A detailed description of the Wisconsin lakes and their plant and animal life is given in Bulletins 27 and 64 of the Wisconsin Geological and Natural History Survey. Only a brief statement will be given here regarding each lake.

Lake Mendota, located at Madison, is 6 miles long, 4 miles wide, and has a maximum depth of 87 feet. It is a hard water lake and contains an abundant growth of plankton.

Green Lake is also a hard water lake, about the same size as Mendota, but has a much greater depth, 225 feet. The plankton growth is somewhat different and also less abundant than in Mendota.

Devil's Lake is a soft water lake, smaller and shallower (depth 43 feet) than Mendota and, compared with the latter, is relatively poor in plankton.

Turtle Lake is located in the cut-over timber region of northern Wisconsin and has highly colored water. It is a soft water lake about the size of Devil's Lake and contains essentially the same type of plankton.

Lake Michigan contains only a small growth of plankton as compared with the Wisconsin lakes. The sample was taken at the North Side Pumping Station intake for the city of Milwaukee. This intake is about 1 mile from shore, and 65 feet below the surface. The depth of the lake at this point is about 300 feet.

Preparation of the Samples.—Surface samples were taken from all of the Wisconsin lakes and a bottom sample from Mendota. The bottom sample was taken 65 feet below the surface where the lake is about 75 feet deep. From 200 to 600 liters of water were brought to the laboratory for analysis. The samples other than those from Mendota were taken in the late fall or winter and were kept cold en route. In most cases less than 10 hours and in no case more than 24 hours elapsed between the time of sampling and the beginning of the analysis. On arriving at the laboratory the sample was immediately run through a high speed Sharples centrifuge at 40,000 R.P.M. which removed the plankton, including 70 to 80 per cent of the bacteria.

The clarified water was concentrated in a vacuum pan at 60°C. to a small volume, 200 to 500 cc., and finally evaporated to dryness with a fan at 65°C. Analyses of the concentrated water from

the vacuum pan and the dried residues showed the same percentage of amino and other forms of nitrogen. It is felt, therefore, that there was no appreciable change in the forms of nitrogen as a result of evaporation of the concentrates to dryness.

Qualitative Test for Proteins and Amino Acids.—Two Mendota samples and one each from Devil's Lake and Green Lake were tested qualitatively for proteins and amino acids by various reagents (4-9). When applied directly to the water concentrate, the reagents did not in general give good tests. Hydrolysis with hydrochloric acid or barium hydroxide hydrolysis and separation of the inorganic salts gave better results. The ninhydrin, Folin and Looney, and Inouye reagents gave strong

TABLE I.
Protein Tests Given by Samples of Concentrated Lake Waters.

Treatment and reagent.	Mendota surface June 18, 1924.	Mendota bottom June 25, 1924.	Devil's Lake Oct. 10, 1923.	Green Lake July 18, 1923.
Gies' biuret.....	Fair.	Strong.	Fair.	Strong.
Millon's, tyrosine.....	"	Good.	Strong.	"
Xanthoproteic.....	"	"	Good.	"
Bromine, tryptophane.....	"	Fair.	Fair.	Fair.
Hopkins-Cole's, tryptophane.....	"	Good.	Strong.	Strong.
Danila's, tryptophane.....	"	"	Fair.	"
May and Rose's, tryptophane.....	"	"	"	"
Inouye's, histidine.....	Good.	Strong.	Good.	"
Harding and Warneford's, ninhydrin.	"	"	Strong.	"
Folin's, amino nitrogen.....	"	"	"	"

color tests on the concentrated water as well as on the hydrolyzed solutions. The results are given in Table I and show that all of the samples gave positive tests with most of the reagents. Positive results with such a number of tests point strongly to the presence of proteins or amino acids in these surface waters.

Quantitative Determination of Certain Amino Acids.

Tryptophane.—This amino acid was determined by the methods of Fürth and Nobel (10), Folin and Looney (11), and May and Rose (7). The first method was modified somewhat by precipitating the tryptophane with mercuric sulfate as in the Folin and Looney procedure. Hydrochloric acid was used to dissolve

the precipitate instead of sodium cyanide, as the latter chemical destroys the color produced by tryptophane with the Fürth and Nobel reagents.

Dried lake samples equivalent to 50 liters of water were hydrolyzed with $\text{Ba}(\text{OH})_2$ (14 gm. $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ per 100 cc.) for 20 hours in an autoclave at 15 pounds pressure ($121^\circ\text{C}.$). Longer heating (30 hours) did not increase the quantity of amino nitrogen. Upon cooling, a large proportion of the inorganic salts settled out. The insoluble material was filtered off, washed with saturated $\text{Ba}(\text{OH})_2$, and the barium removed with 28 per cent H_2SO_4 . The filtrate and washings from the BaSO_4 were concentrated to 50 cc. and the tryptophane determined in 6 cc. aliquots, following the detailed directions of the authors.

As a check on the procedure a known quantity of tryptophane was added to another aliquot of the water sample and the total tryptophane content determined in the usual manner. As complete recovery of the added amino acid was obtained, it is felt that the results are a correct measure of the tryptophane content of the water. The figures obtained by three independent methods check within reasonably close limits.

Tyrosine.—Another aliquot of the hydrolysis products was used for the determination of tyrosine according to the Folin and Looney procedure. That reliable results were secured was attested by the complete recovery of known quantities of tyrosine when added to the unknown solution.

Cystine.—Since cystine is destroyed by heating with alkali, the dried lake water residues equivalent to 20 liters were boiled with 20 per cent HCl for 12 hours instead of $\text{Ba}(\text{OH})_2$. The peptides were completely hydrolyzed at the end of that time as judged by amino nitrogen determinations made during the hydrolysis. The acid was removed in a vacuum and the concentration continued until the volume was less than 50 cc., when the solution was transferred to a 50 cc. flask and made up to volume. Aliquots were analyzed for cystine by the Folin and Looney method. A strong blue color developed in each case and no difficulty was experienced in matching it with the standard. When known quantities of cystine were added to the hydrolyzed samples, the increase in cystine found was exactly equivalent to that added.

Histidine.—Histidine was determined in the acid-hydrolyzed samples by the Koessler and Hanke (12) method as adapted by the authors to urine. Some further modifications were necessary in order to make it applicable to lake water residues. Since tyrosine was present and was found to give a color reaction with the diazobenzenesulfonic acid reagent, it had to be removed completely. Preliminary experiments with solutions containing tyrosine and histidine showed that tyrosine can be separated quantitatively from the histidine by four precipitations with Koessler and Hanke's lead acetate and sodium hydroxide reagents. Rapid sedimentation of the precipitate was obtained

TABLE II.

Quantity of Certain Amino Acids Found in Some Lake Waters.

Amino acid.	Method.	Mendota, surface June 18, 1924.	Mendota bottom June 25, 1924.	Devil's Lake Oct. 10, 1923.	Green Lake July 18, 1923.	Lake Michigan Feb. 28, 1924.	Turtle Lake Jan. 18, 1924.
		mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.	mg. per cu. m.
Tryptophane.	Fürth and Nobel.	10.1	13.1	12.2	14.2	5.5	8.6
"	Folin and Looney.	11.0	14.6	12.9	16.4	7.8	10.6
"	May and Rose.	9.9	12.2		16.1	6.1	
Tyrosine.	Folin and Looney.	10.4	12.5	17.6	9.6	8.3	16.7
Histidine.	Koessler and Hanke.	5.7	10.2	14.8	19.2	6.7	22.7
Cystine.	Folin and Looney.	1.5	6.1	3.3	4.4	2.1	7.5
Total organic nitrogen.....		320	357	177	310	143	487

by the use of a centrifuge. After the lead was removed with disodium hydrogen phosphate the filtrates were concentrated to 2 cc. and the histidine was determined according to the first method given by Koessler and Hanke. The colorimeter readings were exactly the same as those given by untreated solutions of histidine dichloride. Complete recovery was also obtained of known quantities of histidine added to lake samples.

The quantitative data obtained by the methods just described are given in Table II and show that all of the lakes contained appreciable quantities of the four amino acids. The average for tryptophane, tyrosine, and histidine is about 13 mg. and for cystine about 4 mg. per cubic meter of water.

The surface water of Mendota contains less amino acids than does the bottom sample. Certain amino acids are high in some lakes and low in others. No direct comparisons can be made as the samples were not taken at the same time of year nor in the same year. A long series of analyses of Mendota water has shown that there is great variation in the nitrogen content from season to season and from year to year. The type and quantity of plankton growing in the lake are important factors in determining the kind and amount of amino acids in solution at any given time. Since the soluble nitrogen is derived in part from the plankton it is to be expected that the forms of nitrogen will vary.

Other Forms of Nitrogen.

As plenty of material was conveniently available, some other forms of nitrogen were determined on the surface and bottom samples of Lake Mendota.

Free Amino Nitrogen.—The free amino nitrogen was determined on 10 cc. aliquots of the concentrated water by means of the Van Slyke (13) apparatus. For this purpose the regular apparatus was replaced by a special burette which combined the large bulb of the macro apparatus and the capillary tube of the micro apparatus.

Amides.—Aliquots of the concentrated water equivalent to 200 liters of the original water were hydrolyzed for 12 hours with 20 per cent HCl. After hydrolysis the acid was removed in vacuum as directed by Van Slyke. The residue was made alkaline with a 10 per cent suspension of lime and the amide nitrogen distilled off in vacuum at 65°C. into standard acid. The residue in the distilling flask was filtered, washed until free from chlorides, and the humin nitrogen determined in the insoluble material by the Kjeldahl method.

Peptide Nitrogen.—The filtrate and washings were concentrated to a small volume and amino nitrogen was determined in an aliquot by the Van Slyke method. The difference between the amino nitrogen before and after hydrolysis is called peptide nitrogen.

Purines.—The remainder of the solution, after the determination of peptide nitrogen, was used for the determination of total

purines by Benedict and Saiki's (14) modification of Kreuger's method.

Arginine.—After the precipitation of the purines, the filtrate and washings were concentrated in a vacuum to 200 cc. and boiled 6 hours with 40 gm. of KOH in an apparatus similar to that described by Koehler (15). The arginine nitrogen was calculated from the resulting liberated ammonia.

TABLE III.
Forms of Nitrogen in Mendota Water.

No.		Surface June 18, 1924.	Bottom June 25, 1924.
		mg. per cu. m.	mg. per cu. m.
I	Plankton.	92.4	44.9
II	Soluble nitrogen.	515.6	766.9
	1. Free ammonia nitrogen.....	96.0	280.0
	2. Residual ammonia nitrogen...	16.0	20.0
	3. Nitrite nitrogen.....	10.0	17.0
	4. Nitrate "	69.4	92.6
	5. Free amino nitrogen.....	54.0	81.0
	6. Peptide nitrogen.....	135.0	140.4
	7. 1/2 of tryptophane nitrogen...	5.3	7.0
	8. 2/3 of histidine "	3.8	6.8
	9. 3/4 of arginine "	31.1	34.7
	10. Amide nitrogen.....	12.4	19.3
	11. Purine "	8.4	9.5
	12. Amine "	14.2	16.0
III	Forms of soluble nitrogen deter- mined, total.....	455.6	724.3
IV	Undetermined nitrogen.	60.0	41.9

Amines.—Nitrogen in the form of amines was determined by the method of Weber and Wilson (16) in aliquots equivalent to 10 liters of lake water. Since the quantity of ammonia was small it was Nesslerized instead of titrated.

Ammonia, Nitrites, and Nitrates.—These forms of nitrogen were determined directly on the lake water according to the Standard Methods of Water Analysis (17). A small quantity of

ammonia remains in the concentrated water sample which can be obtained by Folin's aspiration method. This is called residual ammonia, while that obtained by simple distillation is called free ammonia.

The complete analyses of the surface and bottom samples of Mendota water are given in Table III. Of the total nitrogen, approximately 90 per cent is accounted for by the different forms determined.

The bottom water runs uniformly higher in all forms of nitrogen than the surface sample. It is conspicuously higher in ammonia, nitrate, and amino nitrogen.

The production of soluble nitrogen takes place principally at the bottom of the lake and is brought about by the action of bacteria on the plant and animal debris which accumulates there. At the time of year when these samples were taken, the lake is stratified into three zones with practically no mixing between the top and bottom layers. In the bottom zone a large production of ammonia and nitrates by nitrifying bacteria and but little consumption of these compounds by plant forms take place. In the surface water the conditions are reversed. The plant and animal life in the bottom stratum is less abundant than in the surface zone and hence the plankton nitrogen is much lower.

The humin nitrogen in the surface and bottom samples amounted to 10.8 and 14.3 mg., respectively. It is approximately the same as that for tryptophane and is in line with the results of Gortner and his associates on the production of humin nitrogen from tryptophane during the hydrolysis of proteins. It is further evidence of the existence of tryptophane in lake waters.

SUMMARY.

The presence of proteins and amino acids in lake waters has been established both by qualitative and quantitative methods. Large samples of water from different types of Wisconsin lakes and also from Lake Michigan were concentrated and tested for proteins by ten different reagents. In all cases positive results were secured. The quantity of tryptophane, tyrosine, histidine, arginine, and cystine was determined in each water. The average of the first three amino acids was about 13 mg. and for cystine about 4 mg. per cubic meter of water.

The quantity of amine, amide, and purine nitrogen was determined in top and bottom water samples from lake Mendota. These forms of organic nitrogen were found to be more abundant in the lower than in the upper stratum.

The soluble nitrogen was separated into twelve different forms and the quantity of each determined. In this way about 90 per cent of the total can be assigned to different fractions.

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A FURTHER REPORT ON IMPARTING ANTIRACHITIC PROPERTIES TO INERT SUBSTANCES BY ULTRA-VIOLET IRRADIATION.

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In a series of communications (1) we have reported experiments which show that antirachitic properties can be imparted to various inert fluids by means of ultra-violet irradiations. In the first report, presented in June, it was recorded that this held true for vegetable oils, such as cottonseed oil. Subsequent to irradiation 0.1 cc. of this oil was sufficient to protect young rats which had been fed on a low phosphorus rickets-producing diet. Later it was found that the same effect could be brought about in growing wheat or in green lettuce leaves—vegetables which possess insignificant protective qualities when they have not been irradiated. Similar results have been reported by Steenbock (2) and his collaborators in regard to vegetable oils and other foods. These investigators used the growth-promoting and bone-calcifying properties of the irradiated food as a criterion of rickets. If growth was brought about on a diet which was complete in all respects excepting for the antirachitic factor it was assumed that the food under investigation possessed antirachitic properties. Throughout our investigations we have taken roentgenologic pictures of the epiphyses and made histologic examinations of the bones, and based our conclusions on these examinations.

Since publishing these reports our investigation has been extended in various directions. It has been found that green wheat which had been irradiated was able to retain its antirachitic power even after it had been stored at ordinary room temperature for a period of 2 weeks. When rats were given 10

gm. per capita daily of this food they were protected to the same extent as when fed the wheat immediately following irradiation. Lettuce, likewise, was found not to lose its potency on being stored for short periods; irradiated green lettuce which was kept for 3 days still maintained its efficacy. Nor was there any distinguishable difference between vegetables which were rich and

TABLE I.
Irradiated Lettuce.

Rat weight.	Rickets-producing diet.	Green or yellow (10 gm.).	Histologic results.	Blood P (inorganic).
gm.				mg.
30-40	Low phosphorus. No. 84.	Green irradiated $\frac{1}{2}$ hr. at 1 ft.	No rickets.	5.26
30-30			" "	
40-46			" "	
32-40			" "	
30-40	" "	Green irradiated $\frac{1}{2}$ hr. at 1 ft. Stored 3 days.	" "	4.50
30-50			" "	
36-48			" "	
41-61	" "	Green (non-irradiated).	Marked "	
34-54			" "	
40-64			" "	
40-60	" "	Yellow irradiated 1 hr. at 1 ft.	No "	2.77
60-80			" "	
44-64			" "	
64-90	" "	Yellow (non-irradiated).	Moderate "	2.26
50-70			" "	
40-70			" "	

those exceptionally poor in chlorophyl. Etiolated wheat, which had been grown in the dark, could, nevertheless, be activated by the ultra-violet rays. Likewise etiolated lettuce leaves, of a pale yellow color, when irradiated with the mercury vapor lamp for a period of 1 hour at a distance of 1 foot, were found to have acquired antirachitic value (Table I). These etiolated plants contained carotinoid pigments, but little if any chlorophyl. Our

experiments with the irradiation of a solution of chlorophyll confirmed these tests, demonstrating that the antirachitic activity of the plants did not depend upon their content of chlorophyll. In this test a 1/5 of 1 per cent watery solution of chlorophyll was used, and each rat was given 0.25 cc. daily. In all these experiments the test material was fed to the animals by pipette and was not incorporated in the diet. In this series of experiments it was found that the rats either were not protected by the addition of irradiated chlorophyll or that the protection was very slight and incomplete.

In the previous communication we reported that human blood serum was not activated by ultra-violet rays. An extension of

TABLE II.
Irradiated Flour.

Rat weight.	Diet.	Histologic results.	Blood P (inorganic).
gm.	per cent		mg. per cent
32-36	Flour (<i>irradiated</i>)..... 95	No rickets.	3.33
38-32	Calcium lactate..... 2.9	" "	
44-40	Sodium chloride..... 2	" "	
30-30	Ferric citrate..... 0.1	" "	
38-36	Flour (<i>non-irradiated</i>)..... 95	Slight "	2.40
40-40	Calcium lactate..... 2.9	Moderate "	
	Sodium chloride..... 2		
30-30	Ferric citrate..... 0.1	Slight "	

this experiment showed that human red blood cells likewise failed to be activated. For this purpose washed human red cells suspended in a small amount of normal salt solution were fed in 0.25 cc. per capita amounts. The cells were disintegrated as the result of being subjected to ultra-violet rays, and the hemoglobin was changed to methemoglobin.

Patent white flour was irradiated. The Sherman-Pappenheimer rickets-producing diet, which was employed throughout these tests, consists of 95 per cent refined flour and 5 per cent of various salts. In this test the flour was irradiated separately for $\frac{1}{2}$ hour and then combined with the salt mixture in the usual ratio of 95 per cent. The result is reproduced in Table II. It will be seen that this dietary has been transformed by means of irradiation.

tion from a markedly rickets-producing to a rickets-protective diet. This activation of flour obviously is of interest from a dietetic point of view. It may also have significance from the standpoint of experimental rickets. It has been the experience in this laboratory, as well as in others, that from time to time the standard diet either fails to produce rickets or produces it to only a slight degree. In the course of the last few years this disconcerting occurrence has been encountered several times. This irregularity, which temporarily has invalidated all experiments, has occurred most often in the spring and summer months. It has never been satisfactorily explained. In view of the possibility of activating flour by means of irradiation, the question arises as to whether such irregularities may be due to differences in the flour brought about by the solar rays. Indeed a consideration of the induction of antirachitic potency in foods by means of light raises the question of the uniformity, from the standpoint of rickets, of various natural foodstuffs which have been used in standard experimental diets.

As stated previously, milk was not activated by irradiation. These experiments are not to be interpreted as indicating that milk cannot be rendered antirachitic by ultra-violet rays, but rather that irradiated milk is unable to confer protection when as little as 0.5 cc. per capita is fed. It is difficult to carry out an experiment on rats with fluid milk; for if we feed large amounts there is the danger of adding sufficient phosphorus to the rickets-producing diet to render it unsuitable from this point of view. Experiments which we have recently carried out would lead us to believe that milk can be activated, and that this property may be evident in infants who, unlike rats, are not protected by a mere increase of the phosphorus in the dietary. Tests with cream, containing 20 per cent of fat, which was irradiated for $\frac{1}{2}$ hour at a distance of 1 foot, resulted in but slight protection from rickets. The fact that cream did not give a definitely positive result leads us to infer that fat is not readily activated by irradiation and that it does not play an essential rôle in the activation of vegetable oils. In this connection it should be emphasized that all our experiments were of a protective and not of a curative nature. This procedure was followed because, in our experience, there are numerous complicating factors,

difficult of interpretation, which may bring about a greater or less degree of healing in bone.

The yolk of egg has been found to be a valuable antirachitic food both for animals and for infants. With these experiences in mind, phosphatide was extracted from egg yolk and irradiated in the usual way. When added to the standard diet in 0.25 cc. amounts this phosphatide was found to have acquired no protective value.

The same was true of glycerol irradiated for $\frac{1}{2}$ hour at 1 foot and fed in 0.1 cc. amounts—the quantity which was efficacious when irradiated oils were given. In spite of this addition, all the rats developed rickets (Table III).

TABLE III.
Irradiated Glycerol.

Rat weight.	Rickets-producing diet.	Amount.	Histologic results (after 28 days)	Remarks.
<i>gm.</i>				
44-40	Low phosphorus. No. 84.	0.1 cc. <i>irradiated</i> $\frac{1}{2}$ hr. at 1 ft.	Moderate rickets.	20 days.
42-46			Slight “	18 “
44-50			Moderate “	
34-36			Slight “	
44-54	“ “	0.1 cc.	Moderate “	
40-40			Slight “	23 “

The investigation of irradiated linseed oil was continued and extended. It was found that this oil had maintained its potency 6 months after it had been irradiated when fed in the same dosage (0.1 cc.) to rats (Table IV). Furthermore, the period of irradiation was successively reduced, first to $\frac{1}{2}$ hour, then to 10 minutes, and finally to 2 minutes, with the mercury vapor lamp set at a distance of 1 foot and operated at 76 volts. Even with the short exposure of 2 minutes the oil was sufficiently activated to prevent entirely the development of rickets, so that the minimal plane of irradiation must lie below this level.

In order to ascertain whether oxidation played a rôle in the activating process, linseed oil was irradiated in an atmosphere of nitrogen. To this end a very small amount of linseed oil was placed in a quartz tube and the air removed from the oil by means

of suction. Nitrogen was then run into the tube, after it had been passed through pyrogallol and over soda-lime. The quartz tube containing the oil was evacuated and flushed with nitrogen several times. The oil was then irradiated for $\frac{1}{2}$ hour at a distance

TABLE IV.
Stored Irradiated Linseed Oil.

Rat weight.	Rickets-producing diet.	Substance fed.	Histologic results.	
gm.				
50-44	Low phosphorus.	0.1 cc. oil, <i>irradiated</i> .	No	rickets.
40-40	No. 84.	Stored 6 mos.	"	"
44-40			"	"
44-50			"	"
32-32	" "	0.1 cc. oil, <i>irradiated</i> .	"	"
34-36		Stored 4 mos.	"	"
34-26	" "	0.25 cc. oil, <i>irradiated</i> .	"	"
26-20		Stored 4 mos.	"	"
30-32	" "	0.25 cc. oil, non-irradiated.	Moderate	"
26-28			"	"
32-40			"	"

TABLE V.
Linseed Oil Irradiated in Nitrogen.

Rat weight.	Rickets-producing diet.	Substance fed.	Histologic results.	Blood P (inorganic).
gm.				mg.
52-50	Low phosphorus.	0.1 cc. oil <i>irradiated</i>	No rickets.	5.43
44-40	No. 84.	$\frac{1}{2}$ hr. at 6 in. in nitrogen.*	" "	
40-42	" "	0.1 cc. oil <i>irradiated</i>	" "	3.71
50-50		$\frac{1}{2}$ hr. at 6 in. in air.*	" "	
52-52			" "	
40-42			" "	

* In quartz tube.

of 6 inches, and 0.1 cc. per capita was fed to the rats. It was found that the lack of oxygen had not prevented activation and that as far as could be judged this oil prevented rickets as well as that which was irradiated in air (Table V).

It has been shown by Zucker (3), and been verified by others, that the active principle of cod liver oil is present in its non-saponifiable fraction and absent in the saponifiable fraction. The experiment therefore suggested itself to ascertain whether this same interrelationship exists in regard to activated vegetable oil. Accordingly, a non-saponifiable fraction was prepared of irradiated and of non-irradiated linseed oil. The oil was saponified by means of NaOH and alcohol, and made into a calcium soap. The unsaponifiable matter was extracted from this by means of acetone. The product was resaponified by means of sodium ethylate, about 3.5 gm. of oily yellow substance being obtained

TABLE VI.

Non-Saponifiable Fraction of Irradiated and Non-Irradiated Linseed Oil.

Rat weight.	Rickets-producing diet.	Fraction tested.	Histologic results.	Blood P (inorganic).
gm.				mg.
38-40	Low phosphorus. No. 84.	0.1 gm. fraction of <i>irradiated oil.</i>	No rickets.	6.09
30-30			" "	
30-29			" "	
30-36	" "	0.1 gm. fraction of non-irradiated oil.	Moderate "	2.79
24-40			" "	
30-35			Slight "	
30-32	" "		Moderate "	1.81
30-32			" "	
24-38			" "	

from a liter of oil. The result of the experiment with this material has been reported briefly in a previous communication (4). It was found that the non-saponifiable fraction of either cottonseed or of linseed oil was unable to protect rats which were placed on the low phosphorus rickets-producing diet. When, however, irradiated linseed oil was fractionated, 0.1 gm. of the non-saponifiable fraction sufficed to confer protection (Table VI). Furthermore, it will be noted that the percentage of inorganic phosphorus in the blood of these rats was more than twice as high as that of similar rats which had received the fraction of the non-irradiated oil. It would seem, therefore, that irradiation had produced a substance similar in its properties to that contained in cod liver oil.

SUMMARY.

Wheat which has been activated by means of ultra-violet irradiation retains its antirachitic potency for a period of weeks. Etiolated yellow wheat as well as green wheat can be rendered active in this way. The same is true of the etiolated (yellow) leaves of lettuce. A solution of chlorophyll is not endowed with antirachitic power by irradiation, nor are hemoglobin, red blood cells, cream, the phosphatide of yolk of egg, or glycerol. On the other hand, refined wheat flour undergoes activation.

Vegetable oil retains its protective power for a period of at least 6 months. It can be activated by an exposure to the mercury vapor lamp for a period of 2 minutes or less. Oxygen plays no rôle in this process as it takes place in an atmosphere of nitrogen.

Fractionization showed that the active principle is present only in the non-saponifiable moiety of the irradiated oil.

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THE ANTIRACHITIC VALUE OF IRRADIATED PHYTOSTEROL AND CHOLESTEROL. I.

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In previous communications it has been shown that vegetable oils acquire antirachitic potency by means of irradiation with the quartz mercury vapor lamp and, furthermore, that this quality is present in the non-saponifiable fraction and lacking in the saponifiable fraction.¹ As the main constituent of the non-saponifiable fraction consists of phytosterol, the next step in this investigation was to ascertain whether phytosterol could be activated by means of irradiation. Accordingly, phytosterol was prepared from cottonseed oil. The unsaponifiable matter from 1 liter of oil was obtained by repeated saponification and dissolving with acetone. This product was then extracted with 95 per cent hot alcohol and the phytosterol repeatedly recrystallized in order to render it as pure as possible. In spite of numerous recrystallizations it could not be rendered absolutely pure. A 1 per cent suspension of this phytosterol in water was irradiated for $\frac{1}{2}$ hour at a distance of 1 foot. It was then fed in 0.25 cc. per capita amounts to rats which were receiving the standard low phosphorus diet. As will be seen from Table I the irradiated preparation conferred protection, whereas the non-irradiated phytosterol did not protect against rickets.

In the same way, experiments were carried out with cholesterol. The cholesterol was a crystalline preparation extracted from brain tissue. It had been repeatedly recrystallized; its crystals were sharply outlined and its melting point was about 147.5°C. These

¹ Hess, A. F., Weinstock, M., and Helman, F. D., *Proc. Soc. Exp. Biol. and Med.*, 1924-25, xxii, 76.

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TABLE I.

Irradiated Cholesterol and Phytosterol.

Rat weight.	Rickets-producing diet.	Substance fed.	Result.	Blood P (inorganic).
gm.				mg. per cent
50-60	Low phosphorus. No. 84.	0.25 cc. cholesterol (1 per cent in water), <i>irradiated</i> 1 hr. at 1 ft.	No rickets.	4.80
44-40			" "	2.99
40-40			" "	
44-34			" "	
50-50			" "	2.85
30-34			" "	
40-40	" "	0.1 cc. cholesterol (1 per cent in water), <i>irradiated</i> ½ hr. at 1 ft.	" "	
40-42			" "	
50-50			" "	
48-50	" "	0.25 cc. cholesterol (1 per cent in water), <i>non-irradiated</i> .	Moderate "	3.64
44-50			" "	
44-50			" "	
40-46			" "	2.41
40-40			" "	
34-38			" "	
44-38	" "	0.25 cc. phytosterol (1 per cent in water), <i>irradiated</i> 1 hr. at 1 ft.	Slight "	3.31
38-30			" "	
40-50			" "	
48-42	" "	0.25 cc. phytosterol (1 per cent in water), <i>non-irradiated</i> .	Moderate "	2.70
50-50			" "	
30-46			" "	
50-54	" "	0.25 cc. phytosterol (1 per cent in water), <i>non-irradiated</i> .	Moderate "	2.40
38-58			" "	
38-36			" "	
40-44	" "	0.25 cc. phytosterol (1 per cent in water), <i>non-irradiated</i> .	Slight "	2.40
30-30			Moderate "	
30-30			Slight "	

crystals were similarly suspended in water in a dilution of 1 per cent and fed in 0.25 cc. amounts. As in the case of the phytosterol it was found that ordinary cholesterol possessed no antirachitic value, whereas the irradiated cholesterol was able to confer absolute protection. Several such experiments were carried out and all gave similar results. In Table I two experiments of this kind are reproduced—one in which 0.1 cc. and another in which 0.25 cc. was fed. The former is equivalent to about 1/150 per cent of the entire daily ration, and indicates how little of the irradiated cholesterol is needed to protect. It is probable that quantitative experiments will demonstrate that even smaller amounts suffice.

Lanolin was irradiated in the same way and 0.25 cc. fed to each rat daily. The non-irradiated lanolin brought about no protection whatsoever. The irradiated lanolin conferred a moderate degree of protection, the animals developing rickets of slight intensity.

The fact that a well defined chemical substance can be endowed with antirachitic potency by means of irradiation is remarkable and of possible physiologic significance. It is particularly interesting that this substance should be cholesterol, which has been regarded as chemically inert, although its ubiquitous presence in the cells has rendered it an object of study for many years. The number of disorders with which, from time to time, it has been associated is manifold, but in no disease or metabolic disturbance has investigation proved it to play a constant or essential rôle.

In regard to the bearing of these results on the pathogenesis of rickets, it is possible to offer merely a hypothesis. As is well known, the epidermal portion of the skin contains a large amount of cholesterol situated in its deeper layers, in close approximation to the prickle cells. It would seem quite possible that the cholesterol in the skin is normally activated by ultra-violet irradiation and rendered antirachitic—that the solar rays and similar artificial radiations are able to bring about this conversion. This point of view regards the superficial skin as an organ which reacts to particular light waves (the epidermal organ) rather than as a mere protective covering. It should be pointed out, however, in connection with this hypothesis that neither cod

liver oil nor its concentrated extract has been found to be efficacious when injected subcutaneously into rachitic animals. Furthermore, the suggested mechanism presupposes not only the formation of active cholesterol within the skin but its further transport by way of the circulation. It would be premature to more than suggest this hypothesis of the action of cholesterol in rickets; its validity must await the results of further investigation.

AMMONIA AND FIXED BASE EXCRETION AFTER THE ADMINISTRATION OF ACID BY VARIOUS PATHS.

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Determination of the additional ammonia excreted in response to administered acid is the only generally useful way of measuring the efficiency of the "ammonia mechanism." Scores of acid administration experiments in which the ammonia excretion was followed have been published, but few of them can be used to calculate the relation between the dose of acid and the amount of ammonia formed to neutralize it. Many of them, it is true, were not planned with this particular object in mind, but even among those that were, more often than not the data are in some important respect inadequate. In some cases the diet was changed in the course of the experiment, in others the after period was omitted, and occasionally the ammonia excretion failed ever to return to its original level—any one of these circumstances is sufficient to deprive an experiment of any value it might otherwise have for the purpose indicated.

Most of the few investigations¹ of the kind that are free from such defects have dealt with the fate of repeated doses of acid over periods of from 3 to 10 days. To this group belong the experiments of Gaetgens² and of Walter³ on the dog, of Lamb and Evvard⁴ on the pig, and of Begun, Herrmann, and Münzer⁵

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¹ Cases that might be considered of a special nature we have left out of account; viz., experiments on pathological subjects, growing animals (including infants), and rabbits.

² Gaetgens, C., *Z. physiol. Chem.*, 1880, iv, 36.

³ Walter, F., *Arch. exp. Path. u. Pharmacol.*, 1877, vii, 148.

⁴ Lamb, A. R., and Evvard, J. M., *J. Biol. Chem.*, 1919, xxxvii, 329.

⁵ Begun, A., Herrmann, R., and Münzer, E., *Biochem. Z.*, 1915, lxxi, 255.

on man. In the last mentioned case, if the body weight is assumed to have been 70 kilos, the daily dose of acid was equivalent on the average to from 9 to 14 cc. of 0.1 N per kilo, the total amount administered being, in one of the two experiments done, 40 cc., and in the other 95 cc., of 0.1 N hydrochloric acid per kilo. In the animal experiments of this group the average daily dose has been between 50 and 90 cc., and the total amount between 160 and 880 cc. of 0.1 N acid per kilo. The experimental conditions, that is to say, have varied over a wide range, but in spite of that, between 60 and 80 per cent of the acid given is in each case accounted for by excess ammonia found in the urine. This fair degree of uniformity stands in marked contrast to the impression given by an uncritical examination of all the experiments of this character that have been published.

As to the fate of single doses, much less is known. Especially careful control is needed in this case, for ordinarily the composition of the urine is altered by the acid during only one 24 hour period. Before feeding acid, and after its effects have disappeared, the ammonia excretion may, of course, vary considerably, more or less in proportion to the total nitrogen. In order that the necessary allowance may be made for this, the ammonia coefficient must be practically constant during the fore and after periods—otherwise the result can be little more than a guess. Unfortunately, hardly anyone who has worked with single doses of acid has presented experiments properly controlled by nitrogen determinations, with a satisfactory degree of regularity in the composition of the urine in the preliminary and final periods, and otherwise suited to the purpose. The only ones, in fact, with any semblance of consistency among themselves are Keeton's⁶ four recent experiments on dogs, fed between 8 and 14 cc. of 0.1 N hydrochloric acid per kilo in one dose. In one of these, according to the author's calculations, all the acid was neutralized by ammonia; in the other three, ammonia accounted for only 30 to 40 per cent of it. In short experiments, ammonia production seems, therefore, to be a less important factor, for the average figure which we estimated from the best experiments of several days duration was 70 per cent.

The apparently subordinate rôle played by ammonia on the

⁶ Keeton, R. W., *J. Biol. Chem.*, 1921, xlix, 411.

1st day of acid administration is a matter of some consequence in connection with the view, for which we can find no valid evidence whatsoever, that man is inferior to Carnivora in his capacity for producing ammonia. This hypothesis, it seems, originated with Limbeck,⁷ and was supposedly confirmed by Camerer⁸—in each case upon wholly inadequate grounds. From more recent statements in support of it—since they seem without exception to be based on comparisons of short experiments on human subjects with the results of prolonged acid feeding to dogs—we are obliged to dissent, inasmuch as the duration of the experiment is to all appearances a vital factor.

Except for the occasional, and certainly not typical, instances⁹ in which administered acid is quantitatively neutralized by ammonia, two other principal mechanisms must be considered in order that the picture may be at all complete. One is the excretion of acid by the kidney (leading to an increase in the titratable acidity of the urine), the other the removal of fixed base from the tissues. The latter is well known to be the chief means of disposing of acid in rabbits; to what extent it occurs in other animals has been debated at intervals for about 50 years, but so far with no wholly satisfactory conclusion. Several investigators^{7,10} have been satisfied to determine the combined weight of sodium and potassium in the form of their chlorides; no accurate quantitative significance can be attached to such results, as there is no way of expressing them in terms of equivalents. Before the development of methods for the determination of total fixed base, the desired information could be obtained in only one way; namely, by determining sodium, potassium, calcium,

⁷ Limbeck, R., *Z. klin. Med.*, 1898, xxxiv, 419. Limbeck claimed that fixed base plays a much greater rôle than ammonia in man. In his experiment, the increase in fixed base reported would have neutralized four times as much acid as was administered, a wholly impossible result.

⁸ Camerer, W., *Z. Biol.*, 1902, xliii, 13. The production of ammonia was in this case, to a large extent, prevented by adding potatoes and other vegetables to the diet on the day when the administration of acid was begun.

⁹ Cf. one of Keeton's experiments, just mentioned, and our own Experiment 2.

¹⁰ Eppinger, H., and Tedesco, F., *Biochem. Z.*, 1909, xvi, 207. Secchi, R., *Biochem. Z.*, 1914, lxxvii, 143.

and magnesium separately. Few have undertaken this, and of those few none has taken pains to secure a fore period showing fair uniformity in the base excretion for at least a few days, with a return to the same level after the effect of the acid has worn off—a very necessary stipulation, and one which it is not at all difficult to meet, as we have found. It is only fair to say that many who have been interested in the influence of acid feeding on the excretion of fixed base, although obliged to use quantitative methods to attack the question, have sought only a qualitative answer to it. It is largely because we have tried to get more explicit information than most previous writers on the subject that we find their data inadequate for our purpose. What calculations we have been able to make from them show, in the great majority of instances, that not far from 20 per cent¹¹ of the acid fed has been neutralized by drawing on the body's supply of fixed base.

In what appear to be the most reliable of the experiments on which this estimate is founded, acid was fed for several days in succession. Under similar conditions, as stated earlier, about 70 per cent of the acid is neutralized by ammonia. There remains, therefore, about 10 per cent to be covered by acid excretion and any other factors that may be involved—among which may be mentioned especially the neutralization of acid by calcium phosphate in the intestine.

If acid were to be fed to a subject on a diet containing a great abundance of phosphate, together with sufficient fixed base to make the urine approximately neutral, there is no doubt that acid excretion could be made to assume a far more important rôle than is ordinarily the case. It is equally certain that an apparent depletion of fixed base could be brought about by giving acid to a subject on a diet having a strongly alkaline ash. In either case, if carried to extremes, ammonia formation would necessarily fall into the background, merely because substitutes for it would be provided in the food. The result, however, could not be accepted as a valid criterion of the body's capacity for producing ammonia. For any real test of the ammonia mecha-

¹¹ Occasionally the figure is higher, but in such cases the diet evidently contained, in proportion to its content of potentially acid substances, a relatively large quantity of fixed base.

nism, it is essential that the tissues shall not be saturated with fixed base beforehand, and also that the acid excretion factor shall, as far as possible, be suppressed. When these provisions have been met, practically the sole alternative to ammonia production is the withdrawal from the tissues of fixed base that constitutes an integral part of them—precisely the occurrence which the ammonia mechanism is evidently designed to prevent—and the way is then open to determine how effective that mechanism may be for the work which it is intended to perform.

Being convinced that dietary influences have been a prolific source of confusion in the past, we decided, in going over this ground which had been covered so many times before, to make one radical departure from the conditions of previous experiments. That is to say, we have used fasting subjects. That many of the uncertainties to which experiments of this nature are liable are thereby at once eliminated will readily be seen. Any fixed base that might be appropriated by acid fed to a fasting animal must previously have been an essential constituent of its tissues, not merely material derived from the food and temporarily stored there. The fasting basis moreover does away, to a very large extent, with the disposal of acid by the process of acid excretion—the titratable acidity of the urine in fasting animals is nearly at its limit anyway, so that acid feeding cannot have much effect upon it unless the buffer content of the urine is increased; and this under the conditions of our experiments did not occur. By the same device one other complication, inevitable when ordinary food is present in the digestive tract, is nearly, if not quite, avoided; namely, the accumulation there of calcium phosphate and other insoluble basic material, which must necessarily react with acid given by mouth and modify its fate to some extent.

Residual Fixed Base.

As a matter of convenience, the total fixed base excretion may be considered as made up of two parts. One of these—often the larger fraction—is represented by base combined as chloride; it is involved, of course, in ordinary salt metabolism, and so is subject to independent variations controlled by the salt intake, the exigencies of osmotic pressure regulation, etc. Of the second fraction a part is combined with weak acids, being distributed

among them in a manner which will vary with the hydrogen ion concentration. Nearly all the rest is in combination with sulfuric acid, which, although a strong electrolyte, differs from hydrochloric acid in being almost wholly produced in the tissues from neutral, or at least less acid, substances.¹²

Hydrochloric acid, in other words, occupies a somewhat unique position among acids in metabolism. Under ordinary conditions it enters the body fully saturated with base, and is eliminated in the same form. Temporarily, to be sure, it may be separated from its base—in the secretion of gastric juice, as well as in the adjustment of membrane equilibrium in the blood, and no doubt elsewhere—and even permanently in connection with such processes as growth and repair¹³ (although, then, too, the gastric cells may effect the separation). But if due allowance is made for these special conditions, distinct advantages are gained by regarding the fixed base combined with all other acids than hydrochloric as a separate entity. In fact, for the proper interpretation of our experimental data, we have found this to be quite necessary, as the following considerations will show.

If feeding sulfuric acid, for example, should increase the excretion of fixed base, and to exactly the same extent the excretion of chloride—substantially what occurred in one of our experiments—it would be necessary to conclude that salt (*i.e.*, chloride) had been removed from the tissues, but that no fixed base had been withdrawn for purposes of neutralization; for the base involved would actually have served no such purpose. To distinguish, in a quantitative way, base that is effective (or potentially so) for neutralizing acid from that which is not, we have subtracted the chloride equivalent from our figures for total fixed base; to the difference it has seemed best to give the non-committal name “residual fixed base.”

¹² The first hydrogen atom of phosphoric acid does not fit accurately into any of these groups. A part, like hydrochloric acid, enters the body and leaves it in combination with base. Some, on the other hand, is set free in the tissues by the hydrolysis of less acid organic compounds, and, in this respect, belongs in a class with sulfuric acid.

¹³ Witness the specific retention of base on refeeding after a period of starvation (Benedict, F. G., *Carnegie Institution of Washington, Pub. No. 203*, 1915, 290. Gamble, J. L., Ross, G. S., and Tisdall, F. F., *J. Biol. Chem.*, 1923, lvii, 633).

The distinction just drawn between base combined with hydrochloric acid and the rest becomes under certain conditions an artificial one. Thus, if hydrochloric acid is introduced into the body, or if alkali is withdrawn from circulation for any purpose (e.g., growth), negative values for the residual fixed base may sometimes be found—meaning, of course, that some hydrochloric acid has been excreted as the ammonium salt. The conception is nevertheless useful in many ways, and in connection with the present problem, as we have said, essential.

The very fact that the residual fixed base can have no definite significance in the case of animals that have been fed hydrochloric acid is a serious drawback to the use of that substance in acid administration experiments. There is no question that the rôle played by fixed base in the process of neutralization can be more accurately followed if some other acid is used. This will explain why we have chosen sulfuric, which had practically been abandoned for the purpose since the work of Gaetgens² in 1880.

EXPERIMENTAL.

The cats which were used in the experiments to be reported were obtained from the animal room, where they had been fed on meat and milk *ad libitum*, and fasted for at least 6 days before the administration of acid. Within this time the relative composition of the urine had become sufficiently constant, as will be seen. The method of handling the cats has been described elsewhere;¹⁴ it will suffice here to say that the bladder was emptied by compressing the abdomen, and that water (100 cc.) was given by stomach tube a few hours before the end of each period in order to increase the accuracy of separation into 24 hour samples.

In the experiments which will first be discussed, the acid (sulfuric) was given *per os*, by means of a catheter of suitable size, used as a stomach tube. The concentration of the sulfuric acid solution was in each instance so adjusted that the requisite quantity of acid could be introduced from a pipette in a volume of exactly 25 cc., and the catheter was then rinsed out with 5 cc. of water.

We have for the present confined our attention to the effect

¹⁴ Fiske, C. H., *J. Biol. Chem.*, 1923, 17, 191.

of single doses of acid—since information on this phase of the problem is particularly scanty—and only comparatively small doses have been used in the work comprising this report. 12.5 to 30 cc. of 0.1 N acid per kilo is the range within which all our present observations lie. The daily acid production in the tissues of fasting cats commonly falls between these limits, so we have not departed farther from natural conditions than necessary to get distinct results.

Total nitrogen was determined by the Kjeldahl method,¹⁵ sulfate volumetrically after precipitation with benzidine,¹⁶ total fixed base by the same method after converting the bases to sulfates,¹⁷ and ammonia¹⁸ and phosphate¹⁹ by colorimetric methods.

The ammonia determinations formed so important a part of the investigation that we preferred to run no risk of decomposition even for the few hours during which the urine was obliged to stand between collection and analysis, and preservation with chloroform in the ice box was adopted as the means least likely to interfere with any of the analytical operations. The chloride determinations in the first two experiments were done according to a modified Volhard method, after destroying the organic matter by fusion with alkali; but the results, as we soon discovered, were too high. We are consequently unable to substantiate the statement²⁰ that dry ashing is a trustworthy procedure under such circumstances. In all subsequent experiments the ashing²¹ was accomplished by boiling with nitric acid and permanganate—after the fashion of the so called von Korányi²² method for blood—and the final titration was done (with thiocyanate) in a way which will be described in full by one of us in collaboration with Dr. K. H. Lin. The essential point in which it differs from the

¹⁵ Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxvii, 461.

¹⁶ Fiske, C. H., *J. Biol. Chem.*, 1921, xlvii, 59.

¹⁷ Fiske, C. H., *J. Biol. Chem.*, 1922, li, 55.

¹⁸ Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 523.

¹⁹ Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.

²⁰ Halverson, J. O., and Schulz, J. A., *J. Am. Chem. Soc.*, 1919, xli, 440.

²¹ Cat urine, even during fasting, contains thiosulfate or some substance resembling it in giving a dark colored precipitate with silver nitrate and nitric acid. Direct titration, without preliminary treatment to destroy this interfering material, is out of the question.

²² von Korányi, A., *Z. klin. Med.*, 1897, xxxiii, 1.

ordinary Volhard titration is that thiocyanate is added until the end-point is nearly, but not quite, reached. The precipitate (AgCl and AgCNS) is then thrown down by centrifugation, and the titration finished on the supernatant liquid. This device we have found to increase the accuracy of the determination several times.

The method by which we have determined how the task of neutralizing acid is distributed between ammonia and fixed base will need a word of explanation.

That variations in the total nitrogen excretion should be taken into account in calculating ammonia production is quite beyond dispute, for reasons stated; an important advantage gained by the use of fasting animals is that fixed base can then be treated in the same manner. During fasting, the chief source of the urinary constituents here involved—aside, of course, from the period during which the animal is under the influence of acid—is muscle. As is well known from observations on other animals, the amount of muscle catabolized during fasting may vary considerably from day to day; and cats, as a rather extensive experience has taught us, are no exception. The total nitrogen excretion, in short fasts at least, is nearly always either rising or falling progressively—and ammonia, sulfate, phosphate, and *residual* fixed base, to mention only the things that now particularly concern us, follow it in whichever direction it may be tending. After the first few days of fasting they all—barring exceptional cases—become and remain proportional to the total nitrogen, within 10 per cent or less. The amounts of both ammonia and residual fixed base which the administration of acid has caused to appear in the urine may then be found by a method of calculation identical in principle with the familiar use of the G:N ratio in experiments on phlorhizinized animals.

The ratios required, *viz.* of ammonia and residual fixed base, respectively, to total nitrogen, have first of all been collected into a separate table (Table I). As an illustration of the method of using them, the case of ammonia production in Experiment 1 may be taken. The ratio (cc. of 0.1 N ammonia per gm. of total nitrogen) was 26 on the day before, and 29 on the day following, the acid period; the average of these two figures (27.5) is, we assume, what it would have been on the intervening day if

TABLE I.
Base-Nitrogen Ratios.

Method of administration...	Per os.						Subcutaneous		Intravenous.	
Dose of acid, cc. 0.1 N per kilo..	30	25			12.5		25		12.5	
Experiment No..	2	1	3	10	4	6	15	16	8	11
Day of fast.	Ammonia (cc. 0.1 N) per gm. nitrogen.									
1	44		27							
2	41		28							
3	34	45	25			21				
4	28	32	27	47		23	26	50	33	26
5	25	32	28	40		23	25	47	32	26
6	24	26	23	34		27	26	50	28	28
7	50*	44*	50*	33		39*	40*	69*	31*	14*
8	26	29	28	54*		30	25	51	19	7
9	22	26	26	40			27			
10		28		43	24					
11				43	24					
12					34*					
13					22					
Residual fixed base (cc. 0.1 N) per gm. nitrogen.										
1			20							
2			26							
3			42			36				
4			35	22		34	28	34	23	24
5			35	29			29	32	34	31
6			39	36		33	30	35	35	36
7			62*	34		36*	54*	65*	54*	176
8			32	38*		30	30	34	35	81
9			35	35			30			
10				29	32					
11					34					
12					48*					
13					37					

* Day of acid administration.

no acid had been given. Subtracting 27.5 from the ratio actually found (44) leaves 16.5 cc. of 0.1 N extra ammonia produced, per gm. of total nitrogen excreted, in response to the sulfuric acid

fed. The total nitrogen on that day happened to be 1.69 gm., from which the total extra ammonia excreted is found to be 1.69×16.5 , or 28 cc. of 0.1 N. Of the acid given, 44 cc. of 0.1 N—as determined by a similar calculation—were recovered from the urine as sulfate; therefore, the fraction neutralized by ammonia was 28/44, or 64 per cent.

In one case only (Experiment 10) the ammonia ratio failed to return to its previous level; in this experiment the figure for ammonia production is naturally less reliable than it would otherwise have been.

Sulfuric Acid per Os.

The analytical results of our oral administration experiments are presented in Tables II to IV, while in Table V will be found the calculations made from them in the manner just described. From the fourth column of Table V it will be apparent that we have in most instances failed to recover all the sulfuric acid that was fed; usually only about 80 per cent of it appeared in the urine as inorganic sulfate.²³ Others who have worked with sulfuric acid have had a similar experience. Whether the acid is incompletely absorbed, or whether the absorption of the last traces of it is merely delayed, failure to reach the circulation promptly is almost certainly the reason for its incomplete recovery, for sulfuric acid injected parenterally reappears in the urine without loss (see below). We have on that account assumed that the amount of sulfate recovered in the urine is identical with what has been absorbed; and upon this, rather than the total dose, we have based our calculations (Table V).

On the average, as this table shows, about 60 per cent of the sulfuric acid given by mouth and subsequently recovered from the urine has been neutralized by ammonia, which is thus stamped as the main protective device under these conditions. There is surely no merit in the contention of Eppinger²⁴—which was never really put to the test of experiment—that fasting animals are incapable of protecting themselves against administered acid.

²³ In the tables, for the sake of uniformity, inorganic sulfate only has been reported. Total sulfate determinations were done as well (except in Experiment 1), and the percentage recovery is the same when figured on that basis.

²⁴ Eppinger, H., *Z. exp. Path. u. Therap.*, 1906, iii, 530.

In three of the experiments (Nos. 1, 3, and 4) in which sulfuric acid was given by mouth, about half of it was neutralized by fixed base. In two others (Nos. 6 and 10) very little increase in fixed base occurred, while in Experiment 2 none at all could be detected (see below). The amount of fixed base available for the neutralization of acid is evidently not the same in different subjects. From this it seems to follow that—with the comparatively small doses which we have used—if acid feeding deprives the body of fixed base at all, it draws only on a supply which is not particularly needed, and which is, therefore, not tenaciously retained. Whether, with larger doses, the ammonia mechanism

TABLE II.

Sulfuric Acid per Os (30 Cc. 0.1 N per Kilo).

Experiment 2. Fasting cat. 2.05 kilos.

Day of fast.	Volume.	Total N.	Inorganic P.	Inorganic sulfate.	Ammonia.	Total fixed base.	Remarks.
	cc.	gm.	mg.	cc. 1 N	cc. 0.1 N	cc. 0.1 N	
2	101	1.74	130		71	54	
3	120	1.77	138		61	63	
4	102	1.82	140		50	72	
5	105	1.85	137		47	69	
6	103	1.97	148	base (47	84	
				20			
7	120	2.08	143	28	104	115	25 cc. 0.245 N H ₂ SO ₄
8	117	1.71	138	48	44	74	(equivalent to 61.3 cc. 0.1 N).

will rise to the occasion and prevent fixed base depletion from reaching serious proportions we hope to learn from further experimentation.

In one experiment (No. 2) apparently the entire burden of neutralization fell on the ammonia mechanism. It was in the first two experiments of the series that the chloride determinations were done by the fusion method, with results that are distinctly too high. In these two cases we are hence unable to give accurate figures for the residual fixed base. The total fixed base was determined, however, and in Experiment 2 it was found to have risen markedly on the day of acid administration (Table II). From this it might be inferred that a considerable part of the acid

TABLE III.

Sulfuric Acid per Os (25 Cc. 0.1 N per Kilo).

Day of fast.	Volume.	Total N.	Inorganic P.	Inorganic sulfate.	Ammonia.	Total fixed base.	Residual fixed base.	Cl	Remarks.
Experiment 1. Fasting cat. 2.11 kilos.									
	cc.	gm.	mg.	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	
3	85	1.45	125	38	65	62			
4	97	1.67	125	46	54	68			
5	98	1.54	115	43	50	60			
6	101	1.51	117	38	39	62			
7	107	1.69	132	86	75	93			25 cc. 0.211 N H ₂ SO ₄ (equivalent to 52.8 cc. 0.1 N).
8	105	1.85	136	50	53	70			
9	81	1.37	115	38	36	69			
10	97	1.55	124	46	44	65			
Experiment 3. Fasting cat. 2.55 kilos.									
2	112	1.74	102	32	49		45	11.7	
3	105	1.44	102	30	36		60	13.8	
4	92	1.23	80	24	33		43	6.8	
5	95	1.20	98	23	33		42	5.1	
6	105	1.13	85	24	26		44	4.4	
7	115	1.13	86	83	57		70	3.6	25 cc. 0.255 N H ₂ SO ₄ (equivalent to 63.8 cc. 0.1 N).
8	80	1.05	83	27	29		34	2.1	
9	87	1.02	71	21	27		36	2.9	
Experiment 10. Fasting cat. 1.12 kilos.									
4	94	0.86	63	17	41		19	6.0	
5	122	0.94	68	19	38		27	7.0	
6	107	1.07	68	24	36		38	17.5	
7	107	1.05	64	24	35		36	16.1	
8	95	0.82	58	40	44		31	7.0	25 cc. 0.112 N H ₂ SO ₄ (equivalent to 28.0 cc. 0.1 N).
9	96	0.82	59	22	33		29	7.4	
10	98	0.80	61	21	34		23	7.8	

TABLE IV.
Sulfuric Acid per Os (12.5 Cc. 0.1 N per Kilo).

Day of fast.	Volume.	Total N.	Inorganic P.	Inorganic sulfate.	Ammonia.	Residual fixed base.	Cl	Remarks.
Experiment 4. Fasting cat. 2.35 kilos.								
	cc.	gm.	mg.	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	
10	102	1.11	87	23	27	39	2.9	
11	92	1.06	75	22	25	36*		
12	113	1.01	76	52	34	54	1.8	25 cc. 0.118 N H ₂ SO ₄ (equiv. alent to 29.5 cc. 0.1 N).
13	96	1.05	80	23	23	39*		

Experiment 6. Fasting cat. 2.75 kilos.

3	115	1.17	90	21	25	42	5.9	25 cc. 0.138 N H ₂ SO ₄ (equiv. alent to 34.5 cc. 0.1 N).
4	125	1.11	79	20	26	38	7.5	
5	85	1.00	61	19	23			
6	83	0.92	66	18	25	30	5.9	
7	103	1.09	63	49	42	39	5.1	
8	94	0.94	69	17	28	28	6.6	

* Owing to shortage of material, the chloride in these two samples was determined only by the fusion method. The figures for residual fixed base are, therefore, too low, to the extent of 2 cc. of 0.1 N or less.

TABLE V.
Neutralization of Sulfuric Acid by Ammonia and Fixed Base.

Dose of acid per kilo.	Method of administration.	Experiment No.	Inorganic sulfate recovered.	Neutralised by		
				Ammonia.	Residual fixed base.	Sum.
cc. 0.1 N			per cent	per cent	per cent	per cent
30	Per os.	2	82	104	(0)*	(104)
25	" "	1	83	64	(46)*	(110)
25	" "	3	88	51	55	106
25	" "	10	75	67	15	82
12.5	" "	4	103	32	42	74
12.5	" "	6	77	60	18	78
25	Subcutaneous.	15	100	34	56	90
25	"	16	100	47	70	117

* Approximate figures, based on chloride determinations made by the fusion method (see text).

was neutralized by fixed base, and that the total extra base eliminated (including ammonia) was far more than the equivalent of the sulfuric acid absorbed. Fortunately the two chloride methods—fusion and the permanganate method which we finally adopted—were compared on more than thirty samples of urine which had been preserved with chloroform. While the error in the former method is considerable, it is always about the same, and for comparative purposes the results are not entirely without value. On the day in question, the chloride output as a matter of fact rose also, and to practically the same extent as the total fixed base. In other words, by the nearest estimate that we are able to make there was no increase in residual fixed base at all. This is perhaps the most striking evidence that we can give to show the importance, really amounting to indispensability, of chloride determinations in connection with such experiments. We have no doubt that the point might have been brought out even more emphatically if we had not used fasting subjects.

In Experiment 1, as nearly as we can tell—since the same inaccurate chloride method was used there also—the increase in residual fixed base excretion was fully enough to cover the fraction of acid not neutralized by ammonia.

With the smallest dose (12.5 cc. of 0.1 N per kilo) the ammonia and fixed base together definitely fall short of covering all the sulfuric acid absorbed (Experiments 4 and 6). Presumably the rest has been disposed of by acid excretion, but the most that we can say on that point is that an increase in titratable acidity *may* have occurred. Acidity titrations were done in nearly all these experiments, but not inconsiderable variations were found in the control periods, and slight changes due to acid feeding cannot readily be detected. If we have not wholly succeeded in our expressed object of eliminating this factor, we have at least reduced it to the point where no estimate of its magnitude can be made.

Parenteral Administration of Sulfuric Acid.

The intravenous administration of acid to cats was tried a few years ago by Bayliss.²⁵ In one of two experiments which he

²⁵ Bayliss, W. M., *J. Physiol.*, 1919-20, liii, 162.

reported, he determined the ammonia in the urine secreted before the injection was begun, and for a period lasting up to 77 minutes after it had been completed. No significant increase was found, and the conclusion was drawn that "ammonia production . . . does not appreciably come into play in short experiments." Although Bayliss made no attempt to control his experiment by using other paths of administration, this has since been done by Keeton, to whose paper we have previously referred.

Keeton, like Bayliss, found little or no increase in the ammonia excretion when acid was given intravenously (to dogs), but when the same dose was introduced *per os* a considerable fraction proved to have been neutralized by ammonia. This seems to show that the method of administration and not the time factor was responsible for Bayliss' result. To explain these findings, Keeton constructed a more or less elaborate hypothesis, the main feature of which is that ammonia is produced only to neutralize acid entering the *portal* circulation or formed in the liver itself.

Hoping to be able to throw some light on this rather curious situation,²⁶ we undertook a number of intravenous injection experiments, using sulfuric acid as before, and at once ran into serious difficulties. One of them had been met before by Bayliss, who found that he could not inject acid into a cat's vein at any speed, no matter how slow, without causing a certain amount of hemolysis. In our experiments, which have been confined to observations on the urine, the same complication has appeared in the form of hemoglobinuria. The actual amount of hemoglobin excreted is small, but with it comes a much larger quantity of albumin—the kidney, in other words, is invariably damaged, and the normal proportionality between total nitrogen and other waste products in the urine disturbed. We were, in consequence, unable to rely upon the method of calculation by means of which in previous experiments we had followed the acid's fate. Our results seem, however, in a rough way, to show: (1) that there is little or no increase in ammonia, and (2) that a sufficient rise in

²⁶ In one respect, Keeton's explanation seems to overshoot the mark. A considerable fraction of the blood that has been deprived of part of its alkali reserve by injecting acid into a peripheral vein is bound to reach the portal circulation. The complete absence of extra ammonia formation in his experiments is not explained by any such theory as he proposes.

residual fixed base excretion occurs to account for all the acid injected, and sometimes much more than enough. The first of these deductions is essentially a confirmation of the *facts* as reported by Keeton; the second, as will be seen, furnished a clue to their interpretation.

Judging by the facility with which hemolysis is produced, cats are unusually susceptible to acid given intravenously, for other species that have been tried rarely show any such effect.²⁷ Further evidence for the abnormal behavior of cats we encountered in distressing abundance, for all our first attempts terminated fatally. We shall not need to describe all the efforts that were made to surmount this difficulty, for a simple and effective way out of it was finally found.

The injections were planned to approximate the rate of absorption when acid—in the minimum dose—is given by mouth. To get a rough idea of what this rate might be, the urine secreted in the first 4½ hours after the administration of acid in Experiment 4 had been collected and analyzed separately—about half the acid had by that time been absorbed and excreted, so much more than half must have entered the circulation. An average rate of 0.05 cc. of 0.1 N acid per kilo per minute was accordingly at first chosen for the intravenous injections.

After several unsuccessful attempts at rates in that vicinity, we discovered that, if the injection was made very slowly (0.015 cc. per kilo per minute) at the start, the rate could gradually be increased at least six or seven times without harm. This was the device adopted in Experiment 8 (Table VI), where we were not troubled even with the hyperpnea which Bayliss was unable to avoid except by the use of morphine. The respiratory rate remained below 40 per minute with the exception of a very short period early in the injection, when it rose to 48. By our usual method of calculation—which, because of the renal injury, is only rough—15 per cent of the sulfuric acid appears to have been neutralized by ammonia, and 90 per cent by fixed base. Whether the ammonia production in this experiment is real or only apparent we can see no way of deciding, but at all events this particular experiment is not seriously inconsistent with Keeton's observations. The next one is.

²⁷ For references to the literature, see Keeton.⁶

When no special precautions are taken to avoid hyperpnea, the story is a very different one. In Experiment 11 we tried—quite unsuccessfully—to control the respiration by making the injection intermittent, the rate varying between 0.04 and 0.07 cc. of 0.1 N acid per kilo per minute. Within the first 20 minutes

TABLE VI.
Sulfuric Acid Intravenously (12.5 Cc. 0.1 N per Kilo).

Day of fast.	Volume.	Total N.	Inorganic P	Inorganic sulfate.	Ammonia.	Residual fixed base.	Cl	Remarks.
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Experiment 8. Fasting cat. 2.14 kilos. Injection started very slowly; no hyperpnea.

	cc.	gm.	mg.	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	
4	87	1.07	77	22	35	25	4.5	
5	106	1.09	83	24	35	37	6.1	
6	88	0.90	73	19	25	31	7.8	
7	129	1.26*	65	46	39	68	18.4	26.7 cc. 0.1 N H ₂ SO ₄ injected in 3½ hrs.
8	90	1.26	85	23	24	44	2.5	

Experiment 11. Fasting cat. 2.58 kilos. Air hunger.

5	100	1.33	101	24	35	41	11.9	
6	63	1.26	100	23	35	45	4.2	
7	82	0.70†	107	55	10	123	35.1	32 cc. 0.1 N H ₂ SO ₄ injected in 4 hrs.
8‡	110	0.90§	55	31	6	73	57.0	

* Non-protein nitrogen. The protein nitrogen was 52 mg.

† Non-protein nitrogen. The protein nitrogen was 79 mg., including 5 mg. in the form of hemoglobin. pH 7.4.

‡ 21 hour urine. The cat died before the end of the period.

§ Non-protein nitrogen. The protein nitrogen was 20 mg.

pronounced air hunger appeared. Although the injection was then entirely suspended for nearly an hour, the respiratory rate remained well above 100 for some time, and was still about 80 (with no sign of falling lower) when the injection was resumed. During the last 140 minutes of the injection—when 75 per cent of the whole dose was introduced at the rate of 0.07 cc. per kilo

per minute—the respiratory rate was never especially high (45 to 57).

The analytical results of this experiment are very instructive (Table VI). It is perfectly clear that, in one sense, the effect of acid was more than compensated for by overventilation, for the extra residual fixed base excreted was about 2.5 times the equivalent of the sulfuric acid injected. With all this fixed base

TABLE VII.
Sulfuric Acid Subcutaneously (25 Cc. 0.1 N per Kilo).

Day of fast.	Volume.	Total N.	Inorganic P.	Inorganic sulfate.	Ammonia.	Residual fixed base.	Cl	Remarks.
Experiment 15. Fasting cat. 2.56 kilos.								
	cc.	gm.	mg.	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	
4	78	1.49	92	20	39	41	5.4	
5	90	1.50	94	20	38	44	5.0	
6	103	1.49	103	18	39	45	5.4	
7	127	1.52	96	86	61	82	4.3	64 cc. 0.1 N H ₂ SO ₄ subcutaneously.
8	98	1.54	91	27	38	46	12.8	
Experiment 16. Fasting cat. 1.59 kilos.								
4	85	0.97	69	25	48	33	2.3	
5	80	0.96	67	26	45	31	3.6	
6	90	0.91	66	25	45	32	3.4	
7	89	0.93	72	65	64	60	2.5	15.9 cc. 0.25 N H ₂ SO ₄ (equivalent to 39.8 cc. 0.1 N) subcutaneously.
8	85	0.85	67	23	43	29	6.8	

set free, it is no wonder that the ammonia excretion actually fell, or that the urine became alkaline. We cannot escape the suspicion that the same compensatory process, in milder form, is the real cause of the apparent absence of ammonia formation when no serious respiratory disturbance takes place. The fact that acid injection at a progressively increasing rate may be borne with safety (as in Experiment 8) is quite possibly to be explained on the same basis.

Having been thus led to doubt the soundness of Keeton's interpretation, we were able to settle the question beyond dispute by means of parenteral administration under conditions less severe. Very slow intravenous injections over prolonged periods we have given no extensive trial, for in cats the vein is then inclined to become clogged by agglutinated corpuscles. For the main point at issue, the precise rate of entrance to the blood stream is of no particular importance, and subcutaneous injection serves the purpose quite as well. Acid given by this path, in a dose of 25 cc. of 0.1 N per kilo (Tables VII and V), was neutralized by ammonia, in Experiments 15 and 16, to the extent of 34 and 47 per cent, respectively. No great significance can be attached to the fact that less ammonia is formed, on the average, than when an equal quantity of acid is given by mouth; with a larger number of experiments the difference might conceivably disappear. The absorption of acid, moreover, is no doubt more rapid from the subcutaneous site, and we cannot be sure that the causes of the peculiar results of intravenous injection are wholly absent here. The formation of ammonia to neutralize acid entering the peripheral circulation is at all events unquestionable.

Influence of Acid on Phosphate Excretion.

An increase in phosphate excretion by the kidney following the feeding of *small* doses of acid, especially to human subjects, has often been observed. Presumably because of its qualitative resemblance to the much more pronounced effect of feeding large quantities of acid for several days or weeks to rabbits,²⁸ it has habitually been ascribed to some process of phosphate mobilization (in the tissues) connected with the regulation of neutrality—nor has the custom been materially interfered with by the presentation of facts which would ordinarily be considered sufficient to abolish it. A corresponding diminution, namely, in the excretion of phosphate by way of the intestine has been demonstrated²⁹—and without evidence to the contrary, based upon actual analytical data including both feces and urine, diversion of phosphate

²⁸ Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113. Goto, K., *J. Biol. Chem.*, 1918, xxxvi, 355.

²⁹ Zucker, T. F., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 272.

from the former to the latter must stand as the accepted explanation.

That the feces lose *all* that the urine gains cannot easily be proved, owing to technical difficulties. It seems to us, therefore, particularly significant that our fasting animals, including those which received acid by paths other than the digestive tract, show no change in phosphate excretion.³⁰

SUMMARY.

When sulfuric acid, in doses of 12.5 to 30 cc. of 0.1 N per kilo, is fed to fasting cats, the greater part of it is usually neutralized by ammonia. The supply of fixed base is drawn upon to a variable extent—from practically none to the equivalent of half the acid absorbed.

The intravenous injection of acid, rapidly enough to cause pronounced hyperpnea, may result in the liberation of fixed base far in excess of what is needed to neutralize all the acid. Under these conditions the ammonia excretion falls.

Probably the same thing happens on a smaller scale if hyperpnea is prevented—as it can be by starting the injection very slowly—for the rate at which acid can be introduced without increasing the rate of respiration becomes progressively greater as the injection proceeds.

That acid need not enter the body by way of the portal circulation in order to stimulate ammonia production is shown by the results of subcutaneous injection.

In none of our experiments has there been any increase in phosphate excretion attributable to the administration of acid.

³⁰ The 13 per cent increase in phosphate excretion in Experiment 1, accompanied by a 12 per cent rise in total nitrogen, clearly has no bearing on the question.

CYSTINE IN THE NUTRITION OF THE GROWING RAT.*

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Osborne and Mendel¹ have shown very clearly that with growing rats on diets containing casein as the sole protein, cystine is the amino acid which first limits the rate of growth when the percentage of protein in the food mixture is reduced below the zone of entire adequacy.

Sure² has drawn a similar conclusion as regards lactalbumin; but his results are much less clear-cut and to us do not seem very conclusive. It is to be hoped that Sure will repeat his work on this point with larger numbers of experiments and a more systematically graded series of diets with diminishing percentages of lactalbumin, as in Osborne and Mendel's work with casein.

We have seen no record of experiments to determine whether cystine is an appreciable factor in the other proteins of milk or in protein-free milk.

Hence it appeared to us desirable to study the question of cystine as a possible "limiting" amino acid in the growth of young rats whose dietary protein is that of cow's milk as a whole; but overdiluted with non-nitrogenous food. This seems of particular interest because of the wide use of growing rats as experimental animals for the study of nutrition and food values, and the frequency with which some of the various forms or products of milk have entered into the dietaries used in such nutrition experiments.

EXPERIMENTAL.

First Series.—In the experiments of the first series the diets consisted essentially of whole milk powder diluted with about

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¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 1.

² Sure, B., *J. Biol. Chem.*, 1920, xliii, 457.

five times its weight of starch, with table salt, and with or without yeast, butter fat, cystine, or ferric citrate as a supplement to find which of these would prove to be limiting factors (for growth of rats) in a diet thus consisting of whole milk overdiluted with starch. The whole milk powder showed on analysis 28 per cent of fat and 4.13 per cent of nitrogen corresponding to 25.8 per cent of total proteins (and amino acids if present). The compositions of the diets as first fed are shown in Table I. The first part of Fig. 1 shows the results of feeding these five diets to well matched rats of the same litter, beginning at the age of 28 days. Within 4 weeks after the beginning of the experiment it was apparent that only the diet containing the yeast was able to support normal growth. Evidently the first requisite (for

TABLE I.

Compositions of Diets Used in Early Part of Experiments of First Series.

Rat No.	Diet composed of						
	Corn-starch.	Whole milk powder.	Sodium chloride.	Yeast.	Butter fat.	Cystine.	Ferric citrate.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
333	80.33	16.39	1.64	1.64			
334	80.33	16.39	1.64		1.64		
335	81.83	16.37	1.64			0.16	
336	81.83	16.37	1.64				0.16
337	81.97	16.39	1.64				

rats) of the diet of milk thus overdiluted with starch is vitamin B. This was then supplied to the diets which had not previously contained it, the diets for the remainder of this experiment becoming as shown in Table II.

The point at which the change was made from the diets of Table I to those of Table II, or, in other words, at which the yeast was given to those rats which had not previously received it, is shown by the short, heavy vertical line cutting the four lower curves in Fig. 1 at a point 4 weeks from the beginning of the experiment.

The yeast fed was a dried brewery yeast which showed on analysis 8.3 per cent of moisture, 7.6 per cent of nitrogen, and 0.44 per cent of sulfur.

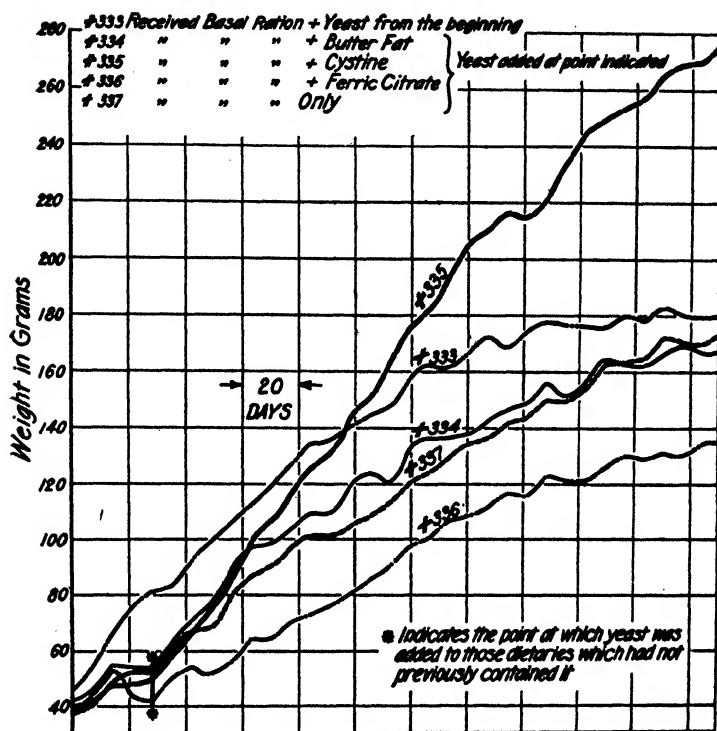


FIG. 1. Growth of young rats from 28 days of age upon diets, the detailed composition of which is given in Tables I and II, showing that when milk is overdiluted with starch vitamin B becomes the first limiting factor and cystine the first limiting amino acid for growth of young rats.

TABLE II.
Compositions of Diets Used in Main Part of Experiments of First Series.

Rat No.	Diet composed of						
	Corn-starch.	Whole milk powder.	Sodium chloride.	Yeast.	Butter fat.	Cystine.	Ferric citrate.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
333	80.33	16.39	1.64	1.64			
334	78.69	16.39	1.64	1.64	1.64		
335	80.17	16.36	1.64	1.64		0.20	
336	80.17	16.36	1.64	1.64			0.20
337	80.33	16.39	1.64	1.64			

When the vitamin B had been supplied by the addition of the yeast, growth was resumed in all cases, but not at a fully normal rate. Rat 335, whose diet contained the added cystine, now made much the most rapid growth of all, gradually overtaking and eventually far outstripping Rat 333, which had made greater growth at the beginning because of having received a diet containing yeast from the start.

It is apparent from Fig. 1 that in these experiments vitamin B was the first requisite and cystine the second; and hence that cystine was the first limiting amino acid of milk as a whole when so diluted with starch as to retard the growth of young rats.

Second Series.—Here ten rats of the same litter and nearly the same initial weight were fed from the age of 31 days, first, two on

TABLE III.

Compositions of Diets Used in Experiments Shown in Fig. 2 and in First Part of Those Shown in Fig. 3 (Second Series of Experiments).

Rat No.	Diet composed of						
	Corn-starch.	Whole milk powder.	Sodium chloride.	Yeast.	Butter fat.	Cystine.	Ferric citrate.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
379, 380	80.67	16.66	1.67	1.00			
381, 382	78.67	16.66	1.67	1.00	2.00		
383, 384	80.50	16.63	1.66	1.00		0.20	
385, 386	80.50	16.63	1.66	1.00			0.20
387, 388	79.67	16.66	1.67	2.00			

each of five diets similar in general character to those used in the first series, but differing in detail as shown in Table III; and, later, 1 per cent of calcium carbonate was added to the diet of one of each two rats to determine whether the extra calcium would increase the rate of growth which, however, did not prove to be the case. Figs. 2 and 3 summarize the results. Here again the growth curves show very clearly that cystine is the "limiting" amino acid for growth of young rats in the proteins of milk as a whole.

SUMMARY AND DISCUSSION.

By experiments in which cystine was added to a diet containing whole milk as the essential source of protein, but overdiluted with

starch, it has here been determined for milk as a whole, as Osborne and Mendel had previously shown for casein, that cystine is the first limiting amino acid of the proteins of cow's milk for the growth of young rats.

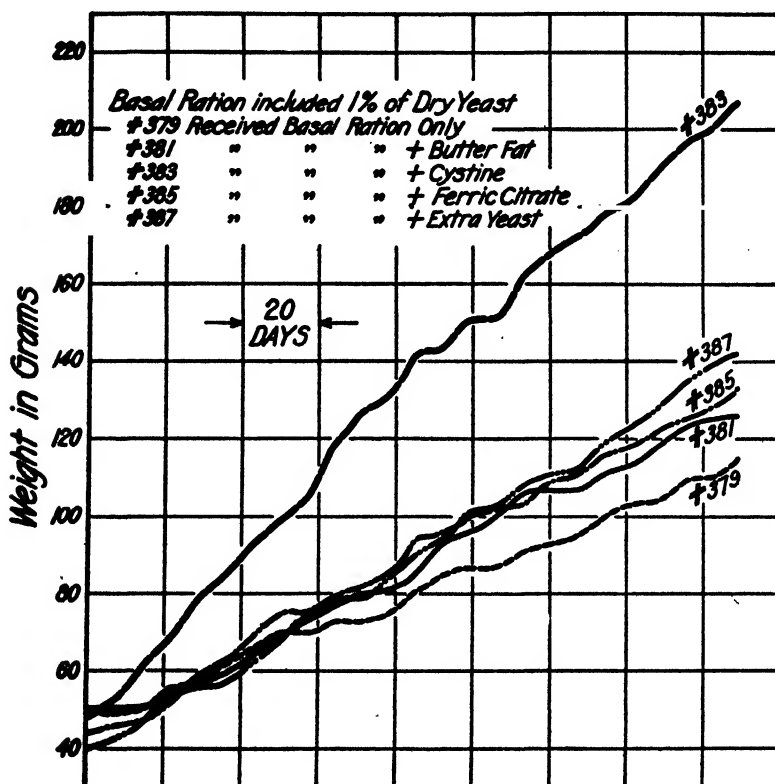


FIG. 2. Growth of young rats on diets given in Table III, showing that under these dietary conditions also cystine was the first limiting amino acid.

It should not be assumed that this would hold equally true if the same diet were fed to a human infant, for while in general the protein metabolism of the rat is strikingly like that of the human, the relative demand for cystine is doubtless much greater in the young rat than in the young child, because the growth of hair is a much more prominent feature in the former case, and this

production of hair must make a relatively heavier demand for cystine than does the growth of body tissue in general.

The sulfur contained in dried brewery yeast when the latter was fed at a level of 1 to 2 per cent of the dry weight of the food

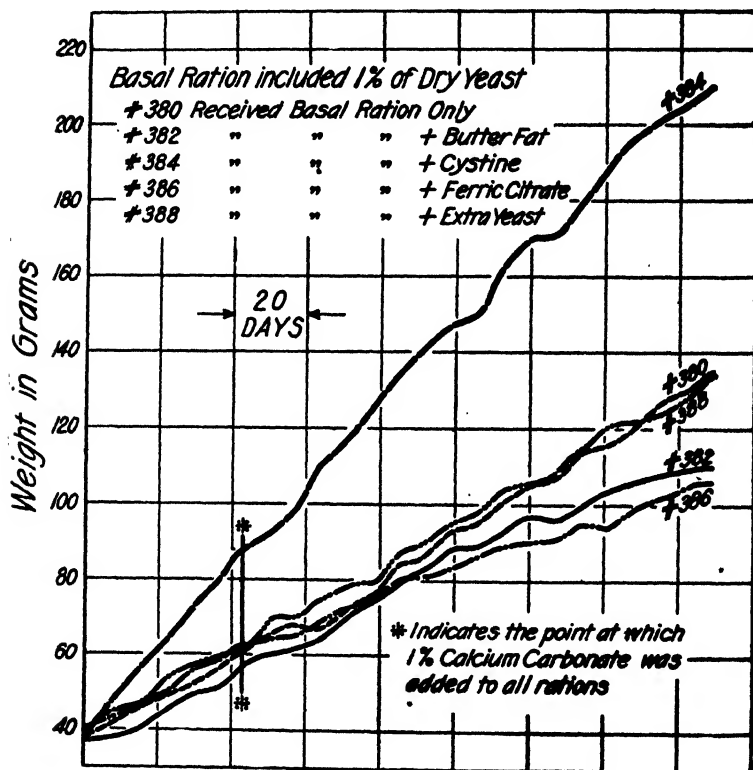


FIG. 3. Growth on diets given in Table III with addition, at point shown, of 1 per cent of calcium carbonate. This added calcium did not increase the rate of growth and here again cystine is shown to be the "limiting" amino acid of cow's milk when overdiluted with starch and used for the nourishment of rapidly growing young rats.

(a proportion ample to furnish the needed supplement of vitamin B) did not have an appreciable effect in these experiments.

The high nutritive efficiency of milk proteins in growth, which has been demonstrated repeatedly by Osborne and Mendel,

Hart, McCollum, Steenbock, and others, is here illustrated anew in that long continued, steady growth at a rate about two-thirds the normal maximum for the age was found to take place upon a food mixture consisting essentially of whole milk slightly supplemented and greatly diluted, and in which diet the total amount of proteins and amino acids was less than 5 per cent.

A study is now in progress in this laboratory in which feeding experiments of this type are being developed into a method for the quantitative determination of cystine.

A NEW TYPE OF PHOSPHORIC ACID COMPOUND ISOLATED FROM BLOOD, WITH SOME REMARKS ON THE EFFECT OF SUBSTITUTION ON THE ROTATION OF *l*-GLYCERIC ACID.*

BY ISIDOR GREENWALD.

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(Received for publication, January 30, 1925.)

The phosphoric acid compounds of the blood may be divided into three groups: protein, lipoid, and acid-soluble (1, 2, 3, 4). In the last are included those compounds that are not precipitated with the proteins and lipoids when the blood is treated with the usual acid protein precipitants.

In plasma, this acid-soluble phosphorus seems to consist almost entirely of inorganic phosphate (3, 4, 5, 6, 7). In fact, it is quite possible that the observed differences between the values obtained for total phosphorus and for inorganic phosphate may be due entirely to errors in the methods employed. But in whole blood, as was first shown by Bloor (3) and confirmed by many others (4, 7, 8, 9, 10), this is almost certainly not the case. The differences between the values obtained for inorganic phosphate and for total phosphorus in the protein- and lipoid-free filtrates are too large to make such a hypothesis acceptable without further evidence.

In 1921, Zucker and Gutman (11) submitted the trichloroacetic acid filtrates from blood to hydrolysis with acids and found that, whereas some of the organically bound phosphoric acid was readily split off, the remainder was not liberated even after prolonged boiling with acids. This they interpreted as evidence of the presence of at least two organic compounds of phosphorus, one of which they termed "non-hydrolyzable."

* A preliminary report was read before the American Chemical Society at Ithaca, N. Y., September, 1924.

Kay and Robison (10) found that all of the phosphorus in the trichloroacetic acid filtrates from blood was liberated as phosphoric acid if the hydrolysis was continued long enough. However, they found that some of the organic phosphoric acid compounds were hydrolyzed by an enzyme present in bones and teeth, particularly in those of young animals. The remainder of the organic phosphoric acid is not decomposed by this enzyme.

More recently, Goodwin and Robison (12) have obtained further evidence of the presence, in human blood, of at least two organic phosphoric acids. One of these forms a barium salt which is insoluble in water and only slightly soluble in cold dilute hydrochloric acid; the other is, apparently, hexose phosphoric acid or a derivative thereof.

The work of Lawaczek (9) also indicates the presence of at least two forms of organically combined phosphoric acid, and, indeed, Lawaczek claims to have isolated, together with Embden and Mayer, derivatives of two compounds. The details as to method and properties are reserved for another publication.

At intervals for several years, vain attempts had been made, in this laboratory, to isolate an organic phosphoric acid from sheep or from beef blood. But the methods employed all proved unsatisfactory. No definite compound was isolated.¹

However, when dog blood was used, unquestionable proof of the presence of an organic phosphoric acid was obtained. This acid was not precipitated by magnesia mixture, but was precipitated by lead acetate and by barium hydroxide. Sufficient quantities of dog blood for the preparation of this substance on an adequately large scale were not available and another source was sought. Pig blood was found to be very satisfactory.

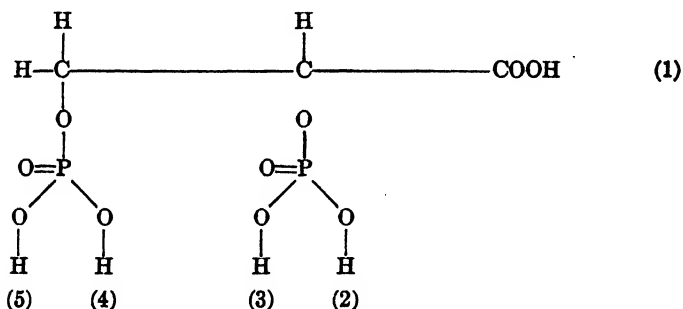
The material was finally obtained in the form of a barium salt, insoluble in water but soluble in dilute hydrochloric acid, and of another barium salt that was precipitated from such acid solution by the addition of alcohol. Analyses of these indicated that the organic phosphoric acid was a compound of either glyceric acid or of an acid of the type of mucic or saccharic acids, in which each

¹ Since writing the above, the barium salt of an organic phosphoric acid has been isolated from sheep blood. It is not the same as the one described in this paper. The method of isolation and the properties of the substance will be described in a subsequent publication.

hydroxyl was combined with a molecule of phosphoric acid. In the salt that was precipitated from aqueous solution by barium hydroxide, the remaining hydrogens of the phosphoric acid groups and the hydrogen of the carboxyl group or groups were all replaced by barium. In the other compound, obtained by precipitation with alcohol, from the acid solution, the carboxyl hydrogen and *one* of the hydrogens of each phosphoric acid group were replaced by barium.

The acid was found to be extraordinarily resistant to acid hydrolysis. It required boiling for days with 5 per cent sulfuric acid to effect complete decomposition. After removal of the sulfuric and phosphoric acids with barium hydroxide and of excess of the latter with carbon dioxide, the filtrate was found to contain a very soluble barium salt, which, upon examination, proved to be the barium salt of levo-glyceric acid.

The formula for the compound is, therefore, $C_3H_5O_8P_2$.



When precipitated from aqueous solution with barium hydroxide all five acid hydrogens are replaced by barium. When precipitated from acid solution by means of alcohol, only the odd numbered hydrogens are replaced. It is proposed that the new substance be known as *diphospho-l-glyceric acid*.

The amount of this substance present in pig blood is very considerable. In different preparations, the purified product isolated represented from 12.2 to 19.3 mg. of phosphorus per 100 cc. of blood and constituted from 30.0 to 41.9 per cent of the total acid-soluble phosphorus, or, at least 36 to 55 per cent of the organic acid-soluble phosphoric acid.

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The same compound has been isolated from human and from dog bloods. The yields have not been so good as from pig blood, probably because of the larger quantity, relative to the total amount, lost in the handling of the smaller quantities.

Of the significance of this substance in metabolism, whether of phosphoric acid, carbohydrate, or fat, we can say nothing. Investigation of this question must, probably, await identification of the other organic phosphoric acids of the blood and the development of suitable methods for their estimation. We are at present so engaged.

It is even possible that this substance is not present in blood as a simple salt, but occurs in the form of an ester or other compound, and that this compound is broken up by the treatment employed for the isolation of the phosphoglyceric acid.

I am indebted to Messrs. F. H. Bergeim and L. Smidth for the determinations of carbon and hydrogen, to Mr. Joseph Gross for the other analyses, and to Mr. Elliot Weier for other assistance.

EXPERIMENTAL.

The blood was defibrinated at the slaughter-house and brought to the laboratory as rapidly as possible, usually within 30 minutes. It was then diluted with 3 volumes of water and the proteins and lipoids were precipitated by the addition of 1 volume of 20 per cent trichloroacetic acid. The mixture was filtered on large fluted papers and the material remaining on the papers was transferred, together with the papers, to large Büchner funnels and there pressed quite dry, with the aid of a sheet of rubber as recommended by Gortner (13).

After removing a sample for the determination of inorganic phosphate (7) and of total phosphorus (oxidation of a considerable quantity with sulfuric and nitric acids, and determination of phosphate on an aliquot by the method of Bell and Doisy (7)), the filtrate was treated with sodium hydroxide until the reaction was no longer acid to Congo red, but only to litmus, and with lead acetate until further addition produced no more precipitate. After standing a few hours, the precipitate was filtered on a Büchner funnel and pressed dry. The filtrate never contained more than a trace of phosphorus.

The precipitate was suspended in water and decomposed with H_2S . The lead sulfide was filtered out and washed. The combined filtrate and washings were aerated to remove the H_2S and were then treated with an excess of magnesium nitrate and ammonium hydroxide. After standing overnight, the precipitate was filtered out and washed with dilute ammonium hydroxide. It was then dissolved in dilute hydrochloric acid, and after addition of a little citric acid to keep the iron that was always present in solution, aliquots were again treated with ammonium hydroxide. The precipitates were filtered on Gooch crucibles and weighed after drying at room temperature and after ignition. The changes in weight indicated that the precipitates were pure magnesium ammonium phosphate. The amounts of phosphorus found in these precipitates were about twice as great as those indicated by the original determinations of inorganic phosphate. In all probability, the increase was due to the decomposition of some labile compound of phosphoric acid.

The filtrate from the first magnesia precipitation was acidified with acetic acid and lead acetate was added to complete precipitation. The lead compound was filtered out and washed thoroughly. Examination of these filtrates showed that they now contained from 2.8 to 5.4 per cent of the total "acid-soluble" phosphorus. Apparently, there had been some change as a result of the previous treatment.

The lead precipitate was again suspended in water and decomposed with H_2S . The lead sulfide was filtered out and washed. The combined filtrate and washings were aerated to remove the H_2S and hot, concentrated barium hydroxide solution was then added until the mixture was alkaline to phenolphthalein and remained so. After standing overnight, this precipitate of the pentabarium salt was filtered out and washed. It dissolved readily in dilute hydrochloric acid, requiring only enough acid to make the reaction distinctly acid to Congo red. From this solution, the tribarium salt was precipitated by means of alcohol. The precipitate was filtered out, redissolved, and reprecipitated and, finally, washed and dried at 100° .

In order to isolate the organic constituent, either the pentabarium or the tribarium salt was suspended in water and digested at 100° with a slight excess of sulfuric acid for at least 24 hours.

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The precipitate was filtered out and washed. The filtrate and washings were evaporated to a conveniently small volume, 5 per cent of sulfuric acid was added and the mixture digested at 100° for several days. After diluting with water, a hot concentrated solution of barium hydroxide was added until the mixture was distinctly alkaline. The mixture of barium sulfate and phosphate was filtered out and washed. The excess of barium hydroxide in the filtrate and washings was removed with CO₂-water, and the filtrate from the barium carbonate was evaporated to a small volume. Addition of alcohol produced a gummy precipitate, but, after resolution and reprecipitation, the barium *l*-glycerate was obtained in the form of microscopic plates. These were dried *in vacuo* over sulfuric acid. When subsequently heated at 100°, the loss in weight was negligible, and there was usually a slight browning. The yield of barium *l*-glycerate was almost calculated.

9.6 gm. Ba₅P₄C₆H₆O₂₀·3H₂O yielded 2.3 gm. purified Ba(C₃H₅O₄)₂· $\frac{1}{2}$ H₂O or 85 per cent of the theoretical.

The specific rotation was, in one case, greater than that recorded in the literature for the dextro-glyceric acid, but, in the other preparations, it was distinctly less. It is believed that some racemization occurred during the preparation. Apparently, treatment with *hot* barium hydroxide should be avoided.

Analyses.

Pentabarium Diphospho-l-Glycerate, Ba₅(P₂C₃H₅O₁₀)₂·3H₂O.

	Ba	P	C	H
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated.....	54.37	9.83	5.70	0.950
Found. From pig blood.....	55.00		5.78	0.976
	53.95	10.3		

Apparently, the compound is apt to be contaminated with the tribarium salt.

Tribarium Diphospho-l-Glycerate, Ba₃ (P₂C₃H₅ O₁₀)₂ · H₂O.

	Ba	P	C	H
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated.....	43.09	12.98	7.53	1.255
Found. From pig blood.....	42.97	12.96	7.52	1.31
“ “ “ “	43.10	12.95	7.57	1.51
“ “ dog “	41.6	13.05		
“ “ human “	40.6	12.6		

Optical Rotation of Diphospho-l-Glyceric Acid.

18 gm. of the pentabarium salt, the equivalent of 7.58 gm. of the free acid, were suspended in water and dissolved by the addition of a minimal quantity of nitric acid. The solution was diluted to 50 cc. In a 2 dm. tube, the rotation was -1.35° .

$$c \text{ (of acid)} = 15.16 \quad [\alpha]_D^{20} = -4.45^{\circ} \quad [M] = -11.8^{\circ}$$

The solution was diluted and the barium was removed with sulfuric acid. The excess of this was removed with lead nitrate. The filtrate from the PbSO₄ was treated with ammonium acetate and lead acetate. The lead precipitate was filtered out and washed thoroughly. It was then decomposed with hydrogen sulfide, and the filtrate from the lead sulfide was diluted to 2,000 cc. 10 cc. portions of this contained 8.14 mg. of phosphorus. The remainder, containing 1.612 gm. of phosphorus or 6.92 gm. of diphospho-l-glyceric acid, was evaporated, *in vacuo*, with a bath temperature not over 35° , to 25 cc. In a 2 dm. tube, the rotation was -1.86° .

$$c \text{ (of acid)} = 27.7 \quad [\alpha]_D^{20} = -3.36^{\circ} \quad [M] = -8.94^{\circ}$$

After adding NaOH until the mixture was just neutralized to Congo red, it was again evaporated to 25 cc., and the rotation determined. In a 2 dm. tube, this was -2.22° .

$$c \text{ (of acid)} = 27.7 \quad [\alpha]_D^{17} = -4.01^{\circ} \quad [M] = -10.65^{\circ}$$

The mixture was then neutralized to thymolphthalein with sodium hydroxide and again evaporated, *in vacuo*, to 25 cc. In a 2 dm. tube, the rotation was -1.51° .

$$c \text{ (of acid)} = 27.7 \quad [\alpha]_D^{21} = -2.73^{\circ} \quad [M] = -7.26^{\circ}$$

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As will be seen later, the molecular rotation of *l*-glyceric acid is not more than -2.44° . It is quite evident from the figures given above that the introduction of the two phosphoric acid groups greatly increases the molecular rotation. The replacement of some of the light hydrogen of the phosphoric acid groups by the heavier sodium led to another increase which was still greater when the replacing cation was barium. Replacement of the carboxyl hydrogen by sodium leads to a lessening of the levorotation, which is similar to the effect observed in the unsubstituted glyceric acid when it is neutralized. This effect of neutralization of the carboxyl group in the diphosphoglyceric acid is sufficient to more than counterbalance the probable increase in levorotation due to the neutralization of the second hydrogen of the phosphoric acid groups.



The analytical combustion of this substance proved to be unexpectedly difficult. At first it was mixed with lead chromate. This gave low results, but subsequent ignition of the boat and contents resulted in a loss in weight which, if calculated as CO_2 , brought the carbon content to the calculated amount. Combustion with a mixture of lead chromate and potassium dichromate in the boat also gave a low value for the carbon content. Treatment of the residue in the boat with sulfuric acid and passage of the evolved gases through the combustion train, which contained lead chromate, yielded an additional amount of CO_2 , making the

	Ba	C	H
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated.....	38.53	20.2	3.11
Found.....	38.5	20.5*	2.95
	38.5	21.4†	3.30
	38.5	21.0‡	

* Combustion with $PbCrO_4$ gave 18.1 per cent; ignition of boat and charge gave 2.4 per cent.

† Combustion with $PbCrO_4$ and $K_2Cr_2O_7$ gave 19.6 per cent; treatment of the residue with H_2SO_4 and passage of the gases through the combustion train gave 2.4 per cent.

‡ Wet combustion.

yield even greater than the calculated. Finally, a wet combustion (14) was performed. The value obtained was slightly too high.

Rotation of Barium l-Glycerate and of l-Glyceric Acid.

The various preparations gave different results, indicating racemization at some stage in the preparation.

Preparation No.	Barium- <i>l</i> -glycerate.			<i>l</i> -Glyceric acid prepared therefrom.		
	<i>c</i>	<i>t</i>	$[\alpha]_D$	<i>c</i>	<i>t</i>	$[\alpha]_D$
		°C.			°C.	
1	9.36	15	+12.2°		Lost.	
2	7.68	20	+ 9.52°	5	20	-1.44°
3	18.48	20	+10.4°	16.1	20	-1.99°
4	9.04	20	+10.7°			

The greatest specific rotation observed by Frankland and Done (15) for the barium salt of *d*-glyceric acid was -10.97° . Neuberg and Silbermann (16) reported -17.38° , but later (17) withdrew this and stated that -10.9° was correct and that their previously reported higher value was due to an error in the setting of the zero point of the instrument. In their earlier report, they had stated that the highest rotation for their barium *l*-glycerate was 8.75° . Calculations from the data they presented show that the same error in the zero point of the instrument would make the specific rotation of their barium *l*-glycerate $+12.1^\circ$.

Assuming that the correct value for the specific rotation of barium *l*-glycerate is 12.2° , Preparation 2 consisted of a mixture of 89 per cent of the salt of the *l* acid and 11 per cent of the *d* acid. From this, the value of $[\alpha]$ for the free *l* acid may be calculated to be -1.85° . Similar calculations for Preparations 3 and 4 indicate that they contained, respectively, 92.7 and 93.9 per cent of the *l* acid. Since these were united approximately in the proportions of 2 parts of Preparation 3 to 1 of Preparation 4 for the determination of the rotation of the free acid, $[\alpha]$ for the pure *l* acid may be calculated to be -2.31° .

However, if $[\alpha]$ for barium *l*-glycerate is 10.97° , the calculated values for the free acid become -1.66° and -2.08° , respectively.

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Frankland and Frew (18) found $[\alpha]$ for their first preparation of the free *d*-glyceric acid to be 2.14° . But since Frankland and Appleyard (19) subsequently reported $[\alpha]$ for the barium salt to be only -10.01° , which value is considerably lower than that later found by Frankland and Done (15) it is probable that the value reported by Frankland and Frew was also too low and that the correct specific rotations are approximately $+12^\circ$ for the barium salt and -2.3° for the free acid.

Distribution of Acid-Soluble Phosphorus in Blood.

Date.	Total.	Inorganic. Bell and Doisy.	In magnesia precipitate.	Isolated as barium phospho- glycerate.	Percentage of total.		
					Inorganic. Bell and Doisy.	In magnesia precipitate.	Isolated as barium phospho- glycerate.
In pig blood.							
1924	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent	per cent	per cent
Mar. 25	44.5	6.32	17.3	19.1	14.2	38.9	41.9
" 18	46.1	5.96	13.1	19.3	12.9	28.4	41.7
Apr. 1	40.7	7.23	16.8	12.2	17.5	41.3	30.0
" 4	41.2	6.04	10.9	16.8	14.7	26.5	41.8
" 23	35.6	6.93	10.4	12.3	19.5	39.2	34.6
May 8	40.9	6.91	10.65	14.6	16.4	26.1	35.7
" 22							
July 4							
In dog blood.							
Dec., 1923 to Mar., 1924	22.8*	4.35*		4.6*			20.0
In human blood.							
							22.0

* Per 100 gm.

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THE STEREOCHEMISTRY OF 2,5-ANHYDROTETROXY-ADIPIC ACIDS.

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INTRODUCTION.

In a previous paper¹ on 2,5-anhydrosugars and their monocarboxylic acids, it was shown that the physical and chemical behavior of these substances could be explained by the rigidity of the molecule, produced by the ring formation between carbon atoms 2 and 5. A more detailed and extensive study of this group should be of considerable assistance in explaining the behavior of normal sugars. Unfortunately, up to date, only one 2,5-anhydrosugar has been prepared in crystalline form and only two monocarboxylic acids have been prepared in the form of their crystalline metallic salts. On the other hand, the dicarboxylic acids, the 2,5-anhydrotetroxyadipic acids, crystallize more readily and are generally more accessible. Some of the properties peculiar to the 2,5-anhydrotetroxycaproic acids should *a priori* also be common to this group of acids. This was actually found to be true in that they show no mutarotation or lactone formation.

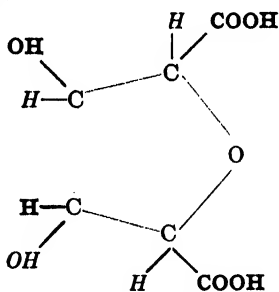
From Figs. 1 to 3 given in the first article and from Figs. 1, 2, and 3 of this paper, it is seen that 2,5-anhydrotetroxyadipic acids may be divided into two groups with respect to the positions of the carboxyls. In one group the two carboxyls are in "*cis*," and in the other in "*trans*," position to each other. The 2,5-anhydrotetroxyadipic acids constitute the first group of substances in which differentiation between the *trans* and *cis* isomers is based on direct evidence and is subject to no uncertainty. These acids should, therefore, offer the most suitable material for testing and

¹ Levene, P. A., *J. Biol. Chem.*, 1924, lix, 135.

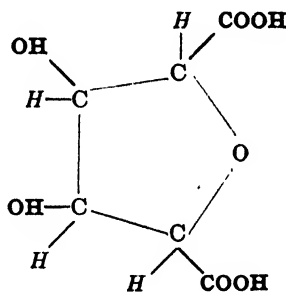
verifying the characteristics generally ascribed to the *trans* and *cis* isomers of the cyclic compounds.

In the present communication the question of the dissociation constants of the *cis* and *trans* acids, on the one hand, and of the normal tetrahydroxy acids, on the other, is discussed. From Tables I and II, it will be seen that all normal acids have nearly the same dissociation constants. In the group of 2,5-anhydro acids, all the *cis* isomers have nearly identical dissociation constants and the *trans* acids also have nearly identical constants. But the constants for the *cis* acids are quite different from those of the *trans* acids.

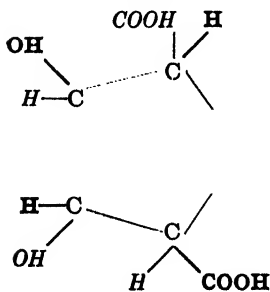
The observed dissociation constants justify the differentiation between the *cis* and *trans* isomers. They, furthermore, permit testing the general theory of the relation between the dissociation constants of a dibasic acid and the distance between the carboxyl groups.



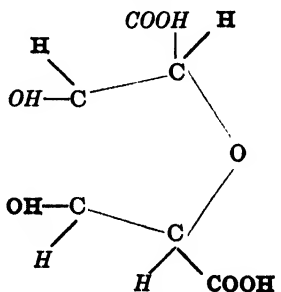
2,5-Anhydrosaccharic acid
(*cis*).



2,5-Anhydromucic acid
(*cis*).



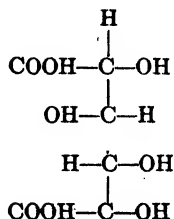
2,5-Anhydromannosaccharic acid
(*trans*).



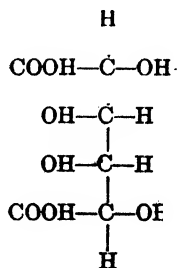
2,5-Anhydroidosaccharic acid
(*trans*).

In the preceding formulas the groups in bold faced type are above the plane of the paper; those in italics are below.

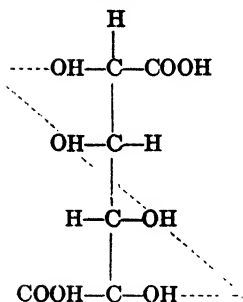
The following modification of the conventional method for writing sugars shows which groups are on the same side of the ring in the *2,5-anhydro* acids.



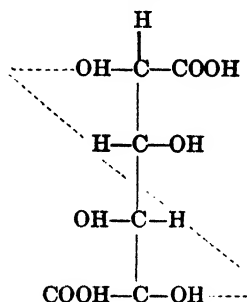
H
Saccharic acid.



Mucic acid.



H
Mannosaccharic acid.



H
Idosaccharic acid.

Figs. 1 and 2 show perspective views of two of these anhydro acids with approximate dimensions in the undissociated molecule, in the monion and in the diion forms.

Structure and Dissociation Constants.

The 2,5-Anhydro Acids.—Bjerrum² has given the formula

$$r = \frac{3.1}{-\log \frac{K_2}{4K_1}}$$

² Bjerrum, N., *Z. physik. Chem.*, 1923, cvi, 219.

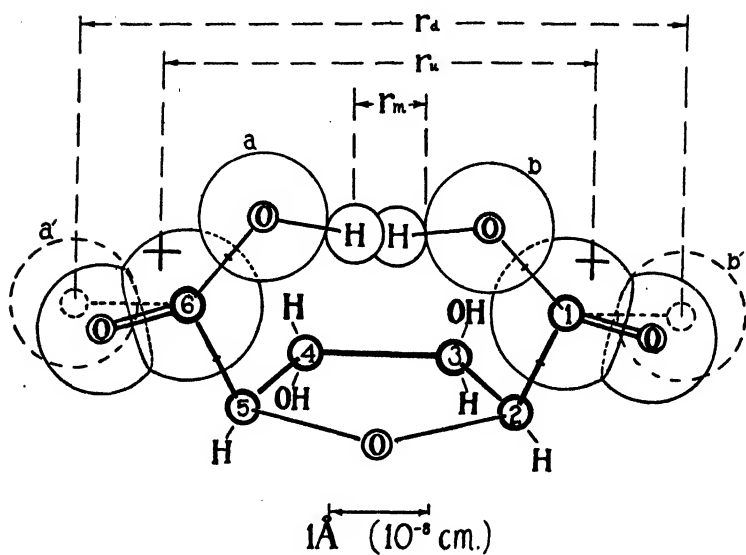


FIG. 1.

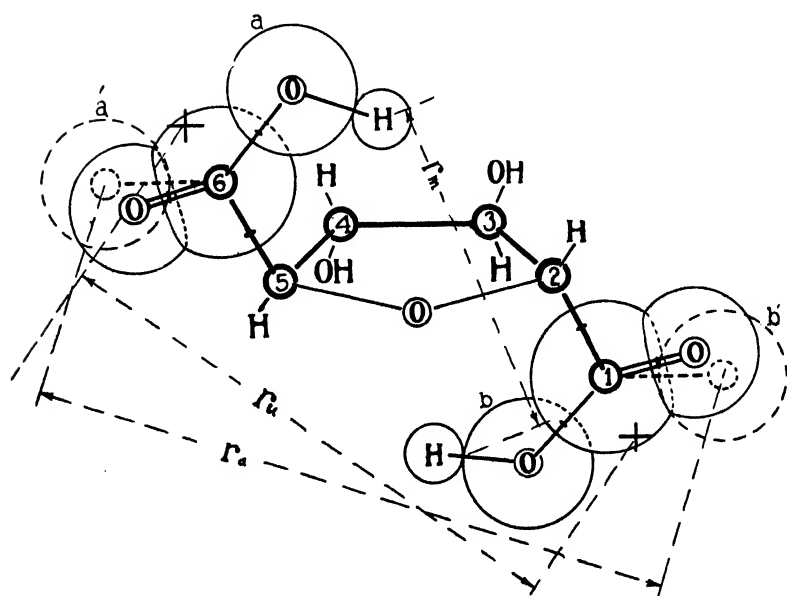


FIG. 2.

FIG. 1. 2,5-Anhydrosaccharic acid (*cis* acid). Except for the positions of the hydroxyls on carbon atoms 3 and 4, this represents the structure of the 2,5-anhydrodicarboxylic acids from glucose, allose, gulose, and galactose.

FIG. 2. 2,5-Anhydromannosaccharic acid (*trans* acid). Except for the positions of the hydroxyls on carbon atoms 3 and 4, this represents the structure of the 2,5-anhydrodicarboxylic acids from mannose, altrose, idose, and talose.

Explanation of Figs. 1 and 2.—The atoms are assumed spherical with the diameters given by Bragg. The angle between valence lines is the tetrahedral angle (109.5°). Only the atoms in the CO·OH groups are represented according to size. The centers of the atoms in the ring are represented by smaller circles joined by lines. The carbon atoms are numbered.

The atoms in the ring lie in a plane almost vertical to the page. The valence lines 1-2 and 5-6 lie in a plane perpendicular to the plane of the ring.

In the undissociated molecule, oxygen atoms *a* and *b* can occupy any position such as *a* or *a'* and *b* or *b'* produced by turning the carboxyl group about the axis 5-6 or 1-2. The crosses give the average position of the H atoms. r_a is the distance between them.

In the monion (singly charged ion), if the attractive force is great enough to bring the remaining H atom and the negative charge to the minimum possible distance, that distance will be r_a .

In the diion (doubly charged ion), if the repulsive force between the two negative charges is great enough to force them to their maximum distance, that distance will be r_a . The charged oxygen atoms will then be at *a'* and *b'*.

to show the relation between the two dissociation constants (K_1 and K_2) and the distance (r) between the two groups in a dibasic acid. His value 3.1 is correct for 18°C. if we assume that the dielectric constant is 80.

The limitations of this formula (due to the work involved in changing the shape of the molecule during ionization) will be pointed out in a future publication. For the present purpose we will give the values of r without ascribing an exact significance to it. Compare with figs. 1 and 2.

TABLE I.

Type.	Acid.	pK ₁	pK ₂	r
<i>Cis.</i>	2,5-Anhydrosaccharic.	1.98	4.94	1.3 Å
	2,5-Anhydromucic.	2.02	4.53	1.6 Å
<i>Trans.</i>	2,5-Anhydromannosaccharic.	2.81	3.80	7.9 Å
	2,5-Anhydroidsaccharic.	3.03	4.00	8.3 Å

TABLE II.

Acid.	pK ₁	pK ₂	r
Saccharic.	3.24	4.12	11 Å
Mannosaccharic.	(3.1)	4.1	(8) Å
Mucic.	3.19	3.99	16 Å
Allomucic.	3.30	4.16	11 Å

Table I gives pK values as determined from electrometric titration and values of r from the formula for 25° C.

$$r = \frac{3.0}{-\log \frac{K_2}{4K_1}} = \frac{3.0}{\text{pM} - 0.6}$$

where $\text{pM} = -\log \frac{K_2}{K_1} = \text{pK}_2 - \text{pK}_1$

and $\text{pM} - 0.6 = -\log \frac{K_2}{4K_1} = \text{pK}_2 - \text{pK}_1 - 0.6$

The values of r in the last column are seen to be much greater in the acids having a *trans* form than in those having a *cis* form.

The Normal Acids.—Similarly the dicarboxylic sugar acids in the normal form (not in anhydride form) are seen to have values of r which are all in the same order of magnitude. See Table II.

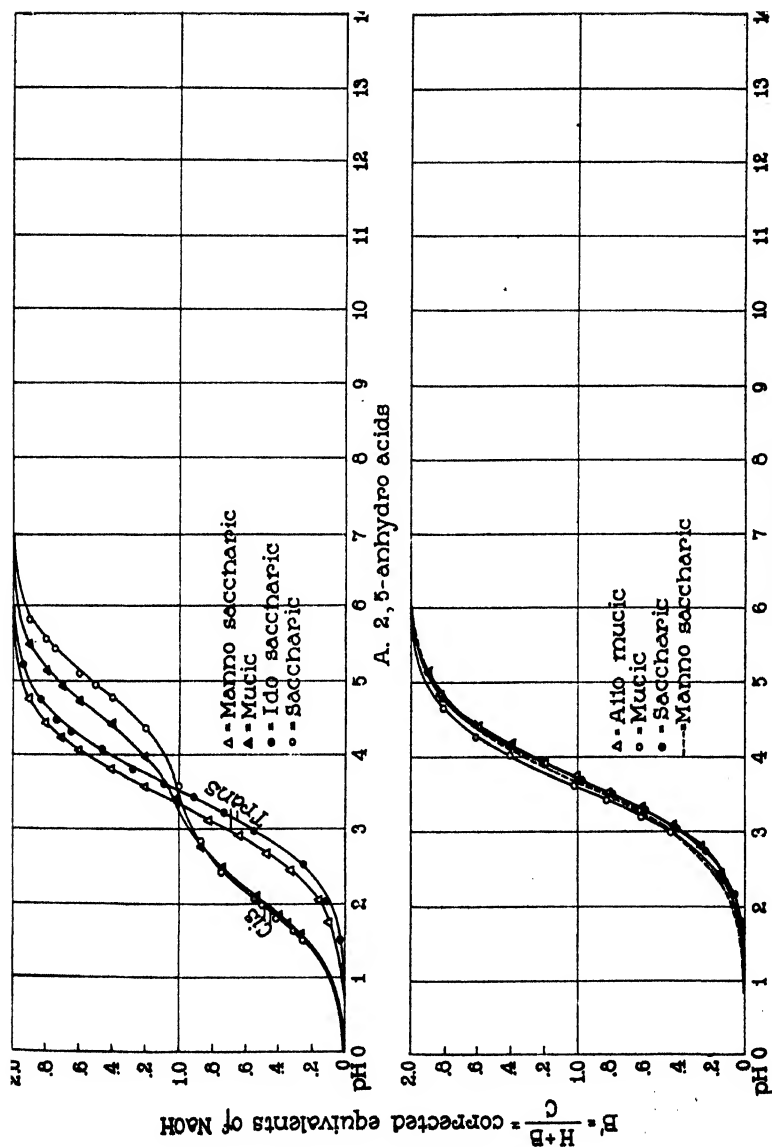


Fig. 3. Titration curves of dicarboxylic sugar acids.

It will be observed that when the pK values are so close together, a small experimental error produces a large difference in the value of r . The normal acids are, therefore, essentially alike. The difference between the *cis* and *trans* acids is, however, so large that there can be no doubt as to the difference in structure. This is shown in Fig. 3.

TABLE III.
Showing Absence of Mutarotation.

Acid.	C	l	Rotation.	
			Initial.	Final in 24 hrs.
2,5-Anhydromannosaccharic.....	0.5	2	+0.51	+0.51
2,5-Anhydrosaccharic.....	1	1	-0.35	-0.36
2,5-Anhydroidosaccharic.....	0.5	2	-0.90	-0.90
2,5-Anhydrotalomucic.....	1	1	+0.29	+0.29

TABLE IV.
Showing Absence of Lactone Formation.

Phenolphthalein used as indicator.

Acid.	Volume of 0.1 M NaOH.	
	Immediately.	After 24 hrs.
	cc.	cc.
2,5-Anhydromannosaccharic.	2.93	2.93
2,5-Anhydroidosaccharic.	2.55	2.55
2,5-Anhydrosaccharic.	4.59	4.59
2,5-Anhydromucic.	4.83	4.83

Therefore, these compounds do not form lactones. This was predicted by use of the structural model.

Mutarotation and Lactone Formation.

From a structural model of the 2,5-anhydro sugar acids it may be seen that they are incapable of forming lactones. The experimental data in Tables III and IV show that this is correct. These acids do not form lactones and do not mutarotate.

The *normal* tetraoxyadipic acids, on the other hand, are capable of lactone formation and are found to mutarotate. Their rates of lactone formation will be discussed in a future paper.

EXPERIMENTAL.

Determination of Dissociation Constants.

The dissociation constants of the eight acids discussed in this article were determined by constant volume electrometric titration in a water-jacketed hydrogen electrode.³ The data are given in Tables V to XI.

The G constants of the acids were determined by the formulas:

$$B' = \frac{H + B}{C} = \alpha_1 + \alpha_2$$

$$G_1 = H \frac{\alpha_1}{1 - \alpha_1} \quad \text{and} \quad G_2 = H \frac{\alpha_2}{1 - \alpha_2}$$

These are related to the K constants by the equations:

$$K_1 = G_1 + G_2 \quad \text{and}$$

$$\frac{1}{K_2} = \frac{1}{G_1} + \frac{1}{G_2}$$

This will be demonstrated in a future publication.

B	=	molar concentration of strong base (NaOH).
$-B$	=	" " " " acid (HCl).
C	=	" " " substance.
H	=	" " " H ion.
B'	=	corrected equivalents of strong base.

In Columns 4, 5, and 6 of Tables V to XI the value of one pG is calculated on the basis of an approximate value of the other pG (the latter being determined graphically). In Columns 7, 8, and 9 the exact value of the other pG is calculated on the basis of the pG value in Column 6. From these pG values the pK values are calculated by use of the formulas given above.

Titration were made at 25.0°C. The pH standard was: 0.10 M HCl = pH 1.040. The potential of saturated KCl junction was assumed constant.

Mannosaccharic Acid.—Four attempts were made to obtain a satisfactory titration of mannosaccharic acid. In spite of modified technique to minimize lactone formation, good results could not be obtained. The pK values are estimated to be approximately 3.1 and 4.1, but the former value may be considerably in error.

³ Simms, H. S., *J. Am. Chem. Soc.*, 1923, **xlv**, 2503.

2,5-Anhydrosaccharic Acid.

Concentration = 0.0250 molar.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} + \frac{H}{C}$	Calculation of pG_2			Calculation of pG_1		
			α_1	$\frac{\alpha_2}{B' - \alpha_1}$	pG_2	α_2	$\frac{\alpha_1}{B' - \alpha_2}$	pG_1
5.81	1.90	1.90	1.00	0.90	(4.86)			
5.55	1.80	1.80	1.00	0.80	4.95			
5.08	1.60	1.60	1.00	0.60	(4.90)			
4.93	1.50	1.50	1.00	0.50	4.93			
4.76	1.40	1.40	1.00	0.40	4.94			
4.36	1.20	1.202	1.00	0.202	4.95			
3.58	1.00	1.010						
2.795	0.80	0.864				0	0.864	1.99
2.442	0.60	0.745				0	0.745	1.98
2.060	-0.20	0.548				0	0.548	1.98
1.808	-0.20	0.42				0	0.42	1.95
1.644	-0.60	0.308				0	0.308	1.99
1.506	-1.00	0.25				0	0.25	1.99
Best average.....					4.94			1.98

$pK_1 = -\log K_1 = 1.98 \quad pK_2 = -\log K_2 = 4.94$

TABLE VI.

2,5-Anhydromucic Acid.

Concentration = 0.0250 molar.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} + \frac{H}{C}$	Calculation of pG_2			Calculation of pG_1		
			α_1 ($pG_1 = 2.00$)	$\frac{\alpha_2}{B' - \alpha_1}$	pG_2	α_2 ($pG_2 = 4.53$)	$\frac{\alpha_1}{B' - \alpha_2}$	pG_1
5.47	1.900	1.900	1.00	0.90	4.52			
5.12	1.800	1.800	1.00	0.80	4.52			
4.92	1.700	1.700	1.00	0.70	4.55			
4.71	1.600	1.600	1.00	0.60	4.53			
4.41	1.400	1.402	1.00	0.40	(4.58)			
3.97	1.200	1.204	0.99	0.214	4.53			
3.39	1.000	1.016	0.962	0.054	(4.62)			
2.77	0.800	0.868				0.017	0.851	2.02
2.47	0.600	0.735				0.010	0.775	2.05
(2.22)	0.400	0.640				0.009	0.631	1.99
2.08	0.200	0.532				0	0.532	2.03
1.835	-0.200	0.395				0	0.395	2.03
(1.727)	-0.400	0.350				0	0.350	2.00
(1.64)	-0.600	0.323				0	0.323	(1.96)
1.572	-0.800	0.272				0	0.272	2.00
Best average.....					4.53			2.02

$pK_1 = -\log K_1 = 2.02 \quad pK_2 = -\log K_2 = 4.53$

2,6-Anhydromannosaccharic Acid.

Concentration = 0.0246 molar.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} = \frac{B}{C} + \frac{H}{C}$	Calculation of pG ₂			Calculation of pG ₁		
			$\frac{\alpha_1}{(pG_2 = 2.92)}$	$\frac{\alpha_2}{B' - \alpha_1}$	pG ₂	$\frac{\alpha_1}{(pG_1 = 3.74)}$	$\frac{\alpha_2}{B' - \alpha_2}$	pG ₁
4.73	1.898	1.898	0.985	0.913	3.71			
4.42	1.797	1.799	0.969	0.830	3.73			
4.20	1.695	1.698	0.951	0.747	3.73			
4.05	1.594	1.598	0.930	0.668	3.74			
3.78	1.391	1.399	0.880	0.519	3.75			
3.56	1.188	1.199	0.815	0.384	3.77			
3.33	0.985	1.004	0.720	0.276	3.75			
3.11	0.782	0.813				0.170	0.643	2.85
2.90	0.579	0.630				0.125	0.515	2.88
2.67	0.376	0.464				0.078	0.386	2.87
2.44	0.173	0.319				0.048	0.271	2.87
2.22	-0.030	0.215				0.030	0.185	2.86
2.03	-0.233	0.146				0.019	0.127	2.87
1.748	-0.639	0.086				0.010	0.076	(2.84)
Best average.....					3.74			2.87

$$pK_1 = -\log K_1 = 2.81$$

$$pK_2 = -\log K_2 = 3.80$$

TABLE VIII.

2,5-Anhydroidosaccharic Acid.

Concentration = 0.0265 molar.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} = \frac{B}{C} + \frac{H}{C}$	Calculation of pG ₁			Calculation of pG ₂		
			$\frac{\alpha_1}{(pG_1 = 3.93)}$	$\frac{\alpha_2}{B' - \alpha_1}$	pG ₁	$\frac{\alpha_1}{(pG_2 = 3.10)}$	$\frac{\alpha_2}{B' - \alpha_2}$	pG ₂
5.19	1.927	1.927				0.991	0.936	(4.03)
4.72	1.833	1.833				0.977	0.856	3.95
4.47	1.740	1.740				0.962	0.778	3.93
4.30	1.642	1.646				0.940	0.706	3.93
4.05	1.456	1.459				0.900	0.559	3.95
3.80	1.256	1.272				0.838	0.434	3.91
3.61	1.077	1.086	0.325	0.761	3.11	0.770	0.316	3.95
3.43	0.888	0.902	0.238	0.664	(3.13)	0.686	0.216	3.98
3.22	0.699	0.722	0.162	0.560	3.10			
2.98	0.509	0.549	0.100	0.449	3.07			
2.52	0.132	0.246	0.037	0.209	3.09			
2.025	0.248	0.110	0.012	0.098	(2.99)			
1.727	-0.623	0.085	0.009	0.076	(2.81)			
1.565	-1.000	0.027	0.008	0.019	(3.29)			
Best average.....					3.10			3.94

$$pK_1 = -\log K_1 = 3.03$$

$$pK_2 = -\log K_2 = 4.00$$

TABLE IX
Saccharic Acid (Monopotassium Salt).

Solution made up 0.0250 molar and found to be 97 per cent pure.
Concentration = $0.0250 \times 0.97 = 0.02425$ molar.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} + \frac{H}{C}$	Calculation of pG ₁ .			Calculation of pG ₂ .		
			$\frac{\alpha_1}{(pG_1 = 4.00)}$	$\frac{\alpha_1}{B' - \alpha_1}$	pG ₁	$\frac{\alpha_1}{(pG_1 = 3.32)}$	$\frac{\alpha_1}{B' - \alpha_1}$	pG ₂
5.13	1.897	1.897				0.985	0.912	(4.12)
4.77	1.815	1.815				0.967	0.848	4.03
4.41	1.608	1.608				0.925	0.683	4.08
4.11	1.402	1.405				0.860	0.545	4.03
3.90	1.196	1.201				0.794	0.407	4.06
3.65	0.990	0.999	0.310	0.689	3.30	0.680	0.317	(3.98)
3.47	0.784	0.797	0.225	0.572	3.35	0.585	0.212	4.04
3.25	0.578	0.600	0.150	0.450	3.34			
2.99	0.371	0.413	0.088	0.325	3.31			
2.71	0.164	0.245	0.050	0.195	3.32			
2.34	-0.04	0.15	0.02	0.13	(3.17)			
2.11	-0.25	0.07	0.01	0.06	3.31			
1.77	-0.70	0.04	<0.01	(0.03)	(3.29)			
Best average.....					3.32			4.04

$$pK_1 = -\log K_1 = 3.24 \quad pK_2 = -\log K_2 = 4.12$$

TABLE X.
Mucic Acid.

Concentration = 0.0250 molar.

The pH of each solution was determined immediately to avoid lactone formation.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} + \frac{H}{C}$	Calculation of pG ₂ .			Calculation of pG ₁ .		
			$\frac{\alpha_1}{(pG_1 = 3.35)}$	$\frac{\alpha_2}{B' - \alpha_1}$	pG ₂	$\frac{\alpha_1}{(pG_1 = 3.89)}$	$\frac{\alpha_1}{B' - \alpha_1}$	pG ₁
4.65	1.800	1.801	0.952	0.849	3.90			
4.27	1.600	1.602	0.893	0.709	3.88			
4.02	1.400	1.404	0.825	0.579	3.88			
3.61	1.000	1.010	0.645	0.365	(3.85)			
3.41	0.800	0.815				0.250	0.565	3.30
3.20	0.600	0.625				0.172	0.453	3.28
2.99	0.400	0.441				0.114	0.327	3.30
2.41	0	0.155				0.032	0.123	3.26
Best average.....					3.89			3.29

$$pK_1 = -\log K_1 = 3.19 \quad pK_2 = -\log K_2 = 3.99$$

TABLE XI.
Allomucic Acid.

Concentration = 0.0250 molar.

Each pH determined immediately to avoid lactone formation.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	$\frac{B}{C}$	$\frac{B'}{C} + \frac{H}{C}$	Calculation of pG ₂ .			Calculation of pG ₁ .		
			$\frac{\alpha_1}{(pG_1 = 3.38)}$	$\frac{\alpha_2}{B' - \alpha_1}$	pG ₂	$\frac{\alpha_2}{(pG_2 = 4.09)}$	$\frac{\alpha_1}{B' - \alpha_2}$	pG ₁
4.81	1.800	1.800	0.963	0.837	4.11			
4.39	1.600	1.602	0.912	0.690	4.04			
4.19	1.400	1.403	0.866	0.537	4.13			
3.93	1.200	1.205	0.782	0.423	4.06	0.410	0.795	3.35
3.75	1.000	1.007	0.700	0.307	4.10	0.315	0.692	3.40
3.51	0.800	0.812	0.575	0.237	(4.02)	0.207	0.605	(3.32)
3.33	0.600	0.619				0.147	0.472	3.38
3.08	0.400	0.433				0.088	0.345	3.36
2.81	0.200	0.262				0.050	0.212	3.38
2.42	0	0.152				0.021	0.131	(3.22)
Best average.....					4.09			3.37

$$pK_1 = -\log K_1 = 3.30 \quad pK_2 = -\log K_2 = 4.16$$

Since the pK values presented here are not corrected for interionic attraction, they are not true constants (and might more properly be designated by pK'). The corrected pK values would be about 0.10 pH unit higher. This does not mitigate the differences between the *cis*, the *trans*, and the *normal* acids.

SUMMARY.

The configuration in space of several dicarboxylic sugar acids was studied by means of: (1) their dissociation constants, (2) their mutarotation, and (3) their lactone formation.

All the *normal* acids are nearly identical in their dissociation.

The *2,5-anhydro* acids neither mutarotate nor form lactones. With regard to dissociation, they fall into two distinct groups. *cis* and *trans*, according to the relative positions of the carboxyls. The members of each group are nearly identical with each other, but differ from the members of the other group.

The results are in entire agreement with those expected by observation of structural models.

HYDROLYSIS OF SUCROSE BY INVERTASE IN THE PRESENCE OF α -METHYL GLUCOSIDE. I.*

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It has been observed by Michaelis and P  chstein (1) and others, that α -methyl glucoside manifests a marked retarding influence on the hydrolysis of sucrose by invertase. The curves given in Fig. 1 represent the hydrolysis of a 10 per cent sucrose solution by invertase in the presence and absence of the glucoside.

Nelson and Hitchcock (2) found that the hydrolysis of 10 per cent sucrose solutions by invertase obtained from brewers' yeast¹ could be represented by the empirical equation:

$$N = \frac{1}{t} \left[\frac{\log 100}{100 - p} + 0.002642 p - 0.00000886 p^2 - 0.000001034 p^3 \right] \quad (1)$$

in which N is a constant, t the time, and p the percentage of sucrose hydrolyzed. By multiplying the value of N by a suitable number, the percentage hydrolyzed-time curves, for different hydrolyses of 10 per cent sucrose solutions, can all be made to superimpose on one another, or in other words, the hydrolyses, irrespective of the amount of invertase used, or the hydrogen ion concentration of the solution, etc., all follow the same course.

It is well known that invert sugar also retards the hydrolysis of sucrose by invertase (3, 4). Since the amount of invert sugar is accumulating in the solution when the sucrose is undergoing hydrolysis, it is quite obvious that its retarding influence is a factor in determining the shape of the hydrolysis curve. Consequently, the parameter N of equation (1) takes this into account as well as the actual rate of hydrolysis at which the sucrose is being hydrolyzed by the enzyme.

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¹ Yeast obtained from Ruppert's Brewery, New York City.

α -Methyl Glucoside Influence on the Course of the Hydrolysis of Sucrose by Invertase.

By applying equation (1) to the data obtained when a 10 per cent sucrose solution is hydrolyzed in the presence of the glucoside, it becomes evident that the calculated values for N , given in Table I, are no longer constant, but show a gradual increase as the reaction progresses. Since N can be considered as a measure of the rate of hydrolysis (5), this increase in its value means that the rate in the case of the hydrolysis in the presence

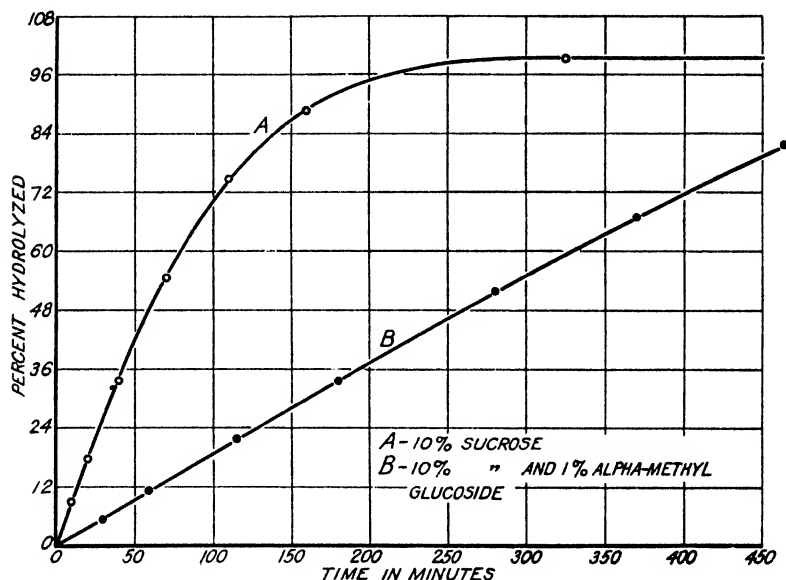


FIG. 1.

of the glucoside gradually increases in magnitude as the reaction proceeds, when compared with the rate of the hydrolysis in the absence of the glucoside. That is to say, if we should attempt to superimpose Curve B, in Fig. 1, representing the hydrolysis in the presence of the glucoside, on Curve A, representing the hydrolysis of the sucrose solution containing no glucoside, by multiplying its ordinates by a number such as to make the initial portions of the two curves coincide, then the greater portion of Curve B would fall above A. The fact that Curve B lies below

Curve A and that N increases during the course of the reaction, when glucoside is present in the solution, means that not only is the reaction slowed up, just as if less invertase had been used, but even the course of the hydrolysis is modified by the presence of the glucoside. This modification of the course of the hydrolysis can also be clearly recognized by comparing the shapes of the two hydrolysis curves.

TABLE I.

Curve A: 10 gm. sucrose per 100 cc. pH = 4.7 Temperature 25°C.

Curve B: 10 " " + 1 gm. α -methyl glucoside per 100 cc.

pH = 4.7. Temperature 25°C. Same amount of invertase in each.

Curve A.				Curve B.			
Time.	Observed rotation.	Change.	N	Time.	Observed rotation.	Change.	N
<i>min.</i>				<i>min.</i>			
0	13.04°			0	16.16°		
10	11.51°	1.53°	644	10	15.84°	0.32°	
20	10.06°	2.98°	642	30	15.24°	0.92°	
40	7.32°	5.72°	639	60	14.26°	1.90°	134
70	3.83°	9.21°	635*	115	12.55°	3.61°	135
110	0.42°	12.62°	641	180	10.51°	5.65°	140
160	-1.91°	14.95°		280	7.47°	8.69°	147
325	-3.64°	16.68°		370	4.93°	11.23°	158
∞	-3.81°	16.85°		435	2.37°	13.79°	173
				605	0.45°	15.71°	
				∞	-0.67°	16.83°	

Values for N only satisfactory between 10 and 80 per cent hydrolysis.

* Probably too low.

Neutralization Effect of α -Methyl Glucoside on the Retardation Due to Invert Sugar.

Since N in equation (1) is proportional to the amount of invertase preparation used, this suggests that the gradual increase in the values for N , given in Table I, might be due to the glucoside having a neutralizing influence upon the retardation of the hydrolysis due to the invert sugar which is gradually accumulating in the solution as the sucrose is being hydrolyzed. Accordingly, hydrolyses were run with sucrose solutions containing α -methyl glucosides in the presence and absence of *added* invert sugar.

TABLE II.

No.	Solution.			Temperature 25°.	
	Sucrose.	Glucoside.	Added invert sugar.	Units of invertase.	pH
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.		
1	5	1	2	1	4.7
2	5	1		1	4.7
3	10	3	12	4	4.7
4	10	3	6	4	4.9
5	10	3		4	4.9
6	2	3		4	4.7
7	2	3	6	4	4.7

No. 1.			No. 2.			No. 3.		
Time.	Rotation.	Change.	Time.	Rotation.	Change.	Time.	Rotation.	Change.
<i>min.</i>			<i>min.</i>			<i>min.</i>		
0	8.89°		0	9.63°		0	17.87°	
5	8.75°	0.14°	5	9.48°	0.15°	10	17.33°	0.54°
10	8.58°	0.31°	10	9.31°	0.32°	20	16.76°	1.11°
20	8.28°	0.61°	20	9.01°	0.62°	30	16.27°	1.60°
45	7.66°	1.23°	40	8.38°	1.25°	60	14.73°	3.14°
75	6.58°	2.31°	75	7.29°	2.34°	100	12.75°	5.12°
120	5.27°	3.62°	120	5.91°	3.72°	160	9.82°	8.05°
210	2.89°	6.00°	210	3.45°	6.18°	240	6.32°	11.55°

No. 4.			No. 5.			No. 6.		
Time.	Rotation.	Change.	Time.	Rotation.	Change.	Time.	Rotation.	Change.
<i>min.</i>			<i>min.</i>			<i>min.</i>		
0	20.29°		0	22.55°		0	12.06°	
10	19.67°	0.62°	10	21.94°	0.61	5	11.81°	0.25°
20	19.13°	1.16°	20	21.41°	1.14°	10	11.58°	0.48°
30	18.66°	1.63°	30	20.92°	1.63°	20	11.11°	0.95°
60	17.04°	3.25°	60	19.30°	3.25°	30	10.63°	1.43°
100	14.90°	5.39°	100	17.17°	5.38°	40	10.17°	1.89°
160	11.96°	8.33°	160	14.16°	8.39°	50	9.77°	2.29°
240	8.30°	11.99°	240	10.34°	12.21°	60	9.38°	2.68°

No. 7.								
Time.	Rotation.	Change.						
<i>min.</i>								
0	9.86°							
10	9.40°	0.46°						
20	8.92°	0.94°						
30	8.51°	1.35°						
40	8.11°	1.75°						
50	7.73°	2.13°						
60	7.38°	2.48°						

The results obtained from these hydrolyses, given in Table II and shown graphically in Fig. 2, show that when moderate concentrations of added invert sugar were employed, there is actually no further increase in retardation beyond that due to the glucoside itself. It is quite evident, therefore, that the retardation of the hydrolysis by invert sugar is greatly reduced by the presence of the α -methyl glucoside.

However, on inspection of Curve 4 in Fig. 2, it can be seen that the glucoside, in this concentration, does not entirely elimi-

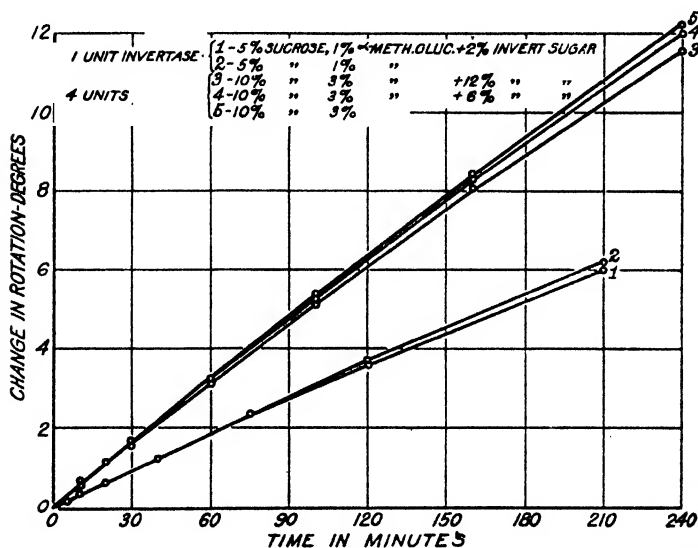


FIG. 2.

nate the retardation due to the invert sugar. After the reaction has progressed to about 50 per cent hydrolysis of the sucrose, the rate begins to drop more in the presence of added invert sugar than in its absence. If this is due to the accumulation of the products of hydrolysis, then if a very high concentration of invert sugar were used, even the initial rate of hydrolysis may be expected to show a lower value. This has been found to be the case from the results obtained when a 10 per cent sucrose solution, containing 3 per cent glucoside and 12 per cent added invert sugar, was hydrolyzed by 4 units of the invertase preparation.

*Influence of Methyl Glucoside on the Retarding Effect of the
Invert Sugar Formed During the Hydrolysis.*

So far we have only discussed the influence of α -methyl glucoside on the retardation induced by invert sugar which had been added to the sucrose solution undergoing hydrolysis. We shall now examine the influence of the glucoside upon the retardation

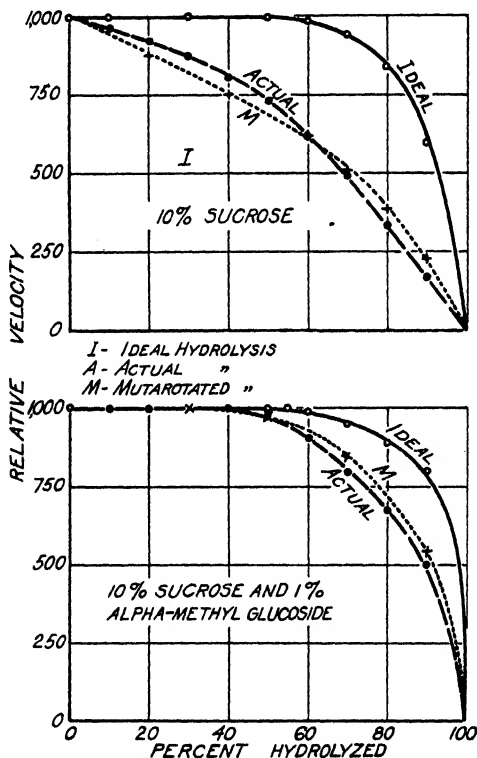


FIG. 3.

caused by the invert sugar formed from the sucrose in the solution, as the latter is hydrolyzed.

The retardation by invert sugar formed in an ordinary hydrolysis of 10 per cent sucrose solution, when no added retardant is present, has been studied by Bodansky (4). His method for determining the effect of invert sugar formed in a hydrolysis

of sucrose has been adopted for studying the effect when α -methyl glucoside is present.

If tangents are drawn to different points on the Curve A, in Fig. 1, representing the course of the hydrolysis of a 10 per cent sucrose solution, then the values of these tangents, Table III, will represent the relative rates at which the sucrose is hydrolyzing at these various stages of the reaction. Thus, if tangents are drawn to the points on the curve corresponding to 10, 20, 30, etc. per cent hydrolysis, then the values of the tangents will correspond to the relative rates of the reaction when the hydrolysis has progressed to 10, 20, 30, etc. per cent of its course. These rates are the relative "actual" rates of hydrolysis and have been plotted, in Fig. 3, both for an ordinary hydrolysis and for a hydrolysis in the presence of α -methyl glucoside.

The rates of hydrolysis, determined in this way and given in Table III, necessarily involve the retarding influence of the invert sugar, for they correspond to different stages of the hydrolysis of the sucrose in the solution. Thus, the tangent to the point on the hydrolysis curve, for a 10 per cent sucrose solution, that shows, for example, 50 per cent of the reaction having been completed, will give the rate of hydrolysis for a solution containing 5 per cent sucrose and approximately 5 per cent invert sugar. This rate, therefore, is a sum of at least two effects—a rate due to the hydrolysis of a 5 per cent sucrose solution, and the cutting down of the rate due to the presence of the 5 per cent invert sugar. In order to determine the magnitude of the retardation due to the invert sugar, it is necessary to know the rate of hydrolysis in the absence of invert sugar and then to compare this rate with that when the invert sugar is present. Just as the tangent to the point on the hydrolysis curve, corresponding to 50 per cent hydrolysis, represents the relative rate of hydrolysis of a solution containing 5 gm. of sucrose and approximately 5 gm. of invert sugar per 100 cc., so the tangent to the curve at its origin would represent the rate of hydrolysis of a solution containing 10 gm. of sucrose and no invert sugar per 100 cc. We shall call this latter rate of hydrolysis, which takes place in the absence of invert sugar, the "Initial rate."

If the initial rates of hydrolysis for several sucrose solutions containing different amounts of sucrose, varying from 10 gm.

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down to say 2 gm. per 100 cc. but no invert sugar, are determined, and then if these initial rates are plotted on a relative basis by putting the maximum rate equal to 1,000, against the varying sucrose concentrations as abscissæ, then a curve like that marked "Ideal" in Part I of Fig. 3 is obtained. This curve has been called ideal in the sense that it, so to speak, represents the course of the hydrolysis of a 10 per cent sucrose solution not influenced by the presence of invert sugar. Or it can be looked upon as the

TABLE III.

Actual rates during the hydrolysis of a solution containing 10 gm. sucrose and 1 gm. glucoside per 100 cc. The rates were obtained from tangents to Curve B in Fig. 1.

Ideal rates for a hypothetical hydrolysis of a 10 per cent sucrose solution containing 1 per cent glucoside. Rates obtained by means of initial rates of hydrolysis for solution containing different concentrations of sucrose, and all containing 1 gm. glucoside per 100 cc.

Percentage hydrolyzed.	Relative rate.	Percentage hydrolyzed.	Relative rate.
0	1,000	0	1,000
10	1,000	50	1,000
20	1,000	55	1,000
30	1,000	60	995
40	1,000	70	950
50	977	80	890
60	904	90	800
70	795		
80	665		
90	498		

Relative rates for "mutarotated" hypothetical hydrolysis of a 10 per cent sucrose solution, containing 1 per cent glucoside. Rates obtained by means of initial rates of hydrolysis for several solutions containing complimentary mixtures of sucrose and added completely mutarotated invert sugar, and all solutions containing 1 gm. glucoside per 100 cc.

Percentage hydrolyzed.....	0	30	50	70	90
Relative rates.....	1,000	1,000	971	850	543

The curves for the rates for 10 per cent sucrose solutions containing no glucoside were taken from Bodansky's paper (4).

course of the hydrolysis of a 10 per cent sucrose solution in which the invert sugar is removed immediately upon its formation, and thus unable to exert any retardation. The ideal curve, in Part II, Fig. 3, was obtained in the same way, the only difference being that besides the various sucrose solutions containing different amounts of sucrose, they all contained 1 gm. of α -methyl glucoside per 100 cc.

The difference between the ordinates of the curves representing the ideal and the actual hydrolysis in the two parts of Fig. 3 shows the extent of retardation exerted by the invert sugar formed during the hydrolysis of a 10 per cent sucrose solution. The curves show that at the beginning of the reaction, when the glucoside is present, the invert sugar hardly retards at all, and that its effect increases as the hydrolysis proceeds. They also show that the retardation due to the invert sugar is always less in the presence of the α -methyl glucoside than in its absence.

The actual rates described above represent rates of hydrolysis of sucrose solutions in the presence of nascent invert sugar that is not completely mutarotated. Bodansky (4) has shown that the rates under these conditions are slightly different from those when the invert sugar is completely mutarotated. In order to see whether this holds when α -methyl glucoside is present, the rates of hydrolysis were determined with solutions of the same composition as those given for the actual rates, but with invert sugar completely mutarotated. The data are given in Table III and shown by Curve M in Fig. 3. They show that freshly formed invert sugar retards differently from completely mutarotated invert sugar, both in the presence and absence of the glucoside.

Relation between the Retardation by Methyl Glucoside and the Concentration of Sucrose.

The curves, given in Fig. 4, represent the relationship between the magnitude of retardation due to α -methyl glucoside and the concentration of sucrose in the absence of invert sugar. The data for these curves were obtained in the same way as that for the ideal curves in Fig. 3; *i.e.*, by the means of initial rates for several series of sucrose solutions, containing different concentrations of sucrose, but a constant amount of the glucoside as indicated in Table IV. First the, change in rotation against time, curves were plotted for each separate hydrolysis, and then the initial rates were obtained from these curves by drawing tangents to their origin. The initial rates thus obtained were compared for each set of experiments, corresponding to each curve in Fig. 4. The greatest velocity in each case was taken

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as 1,000 and the lesser rates as fractions thereof. In this way, all the data were reduced to one scale.

These curves show that the retardation due to the glucoside increases with an increase in sucrose concentration, up to the concentration of the latter when a maximum rate is attained,

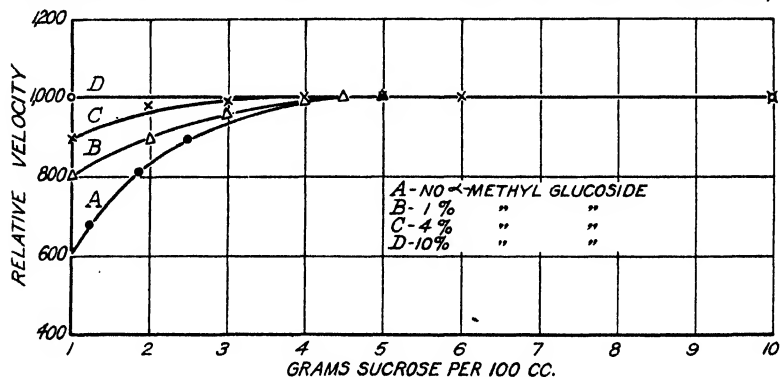


FIG. 4.

TABLE IV.
Data for Curves Shown in Fig. 4.

Curve.								
A	Data taken from paper by Nelson and Bloomfield.*							
B	Sucrose, per cent.	1	2	3	4	4.5	5	10
	Relative rate.	800	890	950	990	1,000	1,000	1,000
C	Sucrose, per cent.	1	2	3	4	6	10	
	Relative rate.	896	980	988	1,000	1,000	1,000	
D	Sucrose, per cent.	1	4	10				
	Relative rate.	1,000	1,000	1,000				

* Nelson and Bloomfield (5), p. 1027.

and in this way suggest another reason, besides the influence of the glucoside upon the retardation due to the invert sugar, discussed in the previous part of this paper, why N of equation (1) should increase, as indicated in Table I, when the equation is applied to the hydrolysis of a 10 per cent sucrose solution in the presence of α -methyl glucoside.

It appears, therefore, that α -methyl glucoside might be exerting two independent influences on the invertase action: the one being the influence on the retardation of the hydrolysis by invert sugar, and the other, the influence on the actual hydrolysis of the sucrose, noticed in the absence of invert sugar. Whether there is any underlying cause common to both of these effects of the glucoside on the hydrolysis of sucrose, the present available data appear to be insufficient to indicate.

Invertase "Hb," used in this study, was obtained from yeast from Ruppert's Brewery, New York City. Its method of preparation is described by Nelson and Hollander (6). The apparatus and procedure followed were essentially those used by Nelson and Bloomfield (7).

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NUTRITIONAL STUDIES OF THE GROWING CHICK.

I. THE RELATION OF SUNLIGHT AND GREEN CLOVER TO LEG WEAKNESS IN CHICKS.*

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The commercial poultryman through years of observation and experience has been taught that to obtain maximum success in the rearing of chicks he must permit his birds to be out of doors on a good range. Fully realizing that nutritional disorders result if his chicks are confined indoors, he uses freshly sprouted grains or stored succulent plant tissue such as roots or cabbage, with the hope of preventing these troubles. He anxiously awaits the advent of warm weather when the birds can be turned out of doors, believing that green plant tissue and soil are necessary for success.

In nature, we observe the young chicks following the hen in search of food with a variable intake of green plant tissue, insects, and soil. Leg weakness of chickens reared under these conditions is rare. Only when the birds are confined indoors, where they do not have access to direct sunlight, green feed, and soil does this malady prevail.

Recent nutritional studies have demonstrated that the chick can be carried to maturity under strict confinement indoors, without direct sunlight, provided a proper diet is furnished (1). However, in the presence of all known dietary factors, but in the absence of the antirachitic factor or its equivalent in the form of radiant energy, not only will abnormal phosphorus and calcium ratios exist in the blood and imperfect skeletal tissue result, but growth will cease (2).

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Hume (3) first suggested the equivalence of fat-soluble vitamins and ultra-violet light in their relation to growth with the belief that light substituted for vitamin A. Steenbock and Nelson (4) have, however, interpreted Hume's data as well as similar data of their own in a different manner. They conclude that ultra-violet light can substitute for the antirachitic factor, but not for vitamin A. Likewise, Goldblatt and Soames (5) have observed the relation of light from a quartz mercury vapor lamp to growth. Also, Powers, Park, and Simmonds (6) recognized the equivalence of light from various sources to the antirachitic properties of cod liver oil as distinct from vitamin A.

Hart, Steenbock, and Lepkovsky (7), studying the relation of light to the growth of chicks, conclude that: ". . . light can play a very important part in the rearing of baby chicks, acting as a supplement or the equivalent to the antirachitic factor of foodstuffs." They further observed from their limited data that: ". . . $\frac{1}{2}$ hour daily exposure to direct sunlight was much more potent in furnishing the antirachitic equivalent than was 5 per cent of a synthetic ration fed as fresh green clover, calculated on the basis of the dry weight of the clover."

The antirachitic properties of green plant tissues have been studied by Shipley, Kinney, and McCollum (8). In feeding rats, suffering from rickets, a ration fortified with the ether extracts of the alfalfa and clover plants at the rate of 250 gm. to a kilo of the ration, these investigators obtained some calcification of the bone tissues in 7 days with complete healing in 33 days. Hart, Steenbock, and Hoppert (9), likewise, suggest the occurrence of the antirachitic factor in green plant tissue. Further evidence of the beneficial effects of green plants in promoting proper bone formation in the pig has been demonstrated by Bohstedt and Robison.¹

While we recognize the occurrence of the antirachitic factor in green plant tissue and the common use by poultrymen of greens, yet no adequate and complete data were available on the quantitative relation in respect to the antirachitic properties of sunlight as compared with green plant tissue. Accordingly, we attempted to make some observations in this regard and determine, if pos-

¹ Personal communication.

sible, the interrelationship of sunlight and green plant tissue (green clover) in the growth of chicks.

EXPERIMENTAL.

Since we were attempting to study the problem from the vitamin standpoint, it was necessary to use a basal ration which was made, as far as possible, complete in all respects, except for the fat-soluble vitamins. Consequently we chose a basal ration of (ground white corn, 2; standard wheat middlings, 1) 80; casein, 16; and salt mixture, 4;² which ration from previous experience had proved to be relatively low in fat-soluble A and the anti-rachitic factor. This ration invariably causes baby chicks to fail between the 4th and 6th weeks. The failure is manifested in a cessation in growth leading to symptoms of leg weakness, although frequently some birds die suddenly without showing severe rachitic symptoms.

On June 3, 1924, 1 day old White Leghorn chicks, hatched from eggs laid by hens of the Station flock and of the same breeding and nutritional history, were divided into fourteen lots and fed the basal ration supplemented in various ways. In each case the chicks received distilled water to drink.

Lots 1 to 10, inclusive, were housed in the poultry building in a floor space of 2×6 feet, covered with pine shavings. Direct sunlight was not accessible. The room was dimly lighted by closed windows, covered with muslin. The remaining four lots (Nos. 11 to 14, inclusive) were housed in a shed supplied with gas heat and allowed a raised runway of boards, screened with wire netting—allowing all-day exposure to sunlight, but preventing access to extraneous food materials. The floors of the brooder and feeding quarters were covered with pine shavings.

Lots 1, 4, and 5 received a ration composed of 3 parts by weight of basal ration and 1 part of green (fresh) red clover. The two latter groups (Nos. 4 and 5) received, in addition to the ration, a daily exposure of $\frac{1}{2}$ and 1 hour to direct sunlight, respectively (excepting Sundays). Lots 2, 6, and 7 received 3 parts of basal

² Salt mixture.

	per cent
Bone ash.....	60
Calcium carbonate.....	20
Sodium chloride.....	20

ration plus 2 parts of green clover. As a further supplement Lots 6 and 7 were exposed to direct sunlight daily (except Sundays) for $\frac{1}{2}$ and 1 hour, respectively. Lots 3, 8, and 9 received equal weights of basal ration and green clover. Lots 8 and 9 received as an additional supplement a daily exposure (except Sundays) of $\frac{1}{2}$ and 1 hour to direct sunlight. Lot 10 received the basal ration and chopped green clover *ad libitum*. At the end of the 6th week this lot was equally divided into two lots, and the one designated as No. 10 *a* was given a daily exposure (except Sundays) of $\frac{1}{2}$ hour to sunlight in addition to the ration. The birds in the other lot (No. 10 *b*) were allowed to continue as before. Lots 11 and 14 received finely chopped green clover *ad libitum* in addition to the basal ration. Soil, which was renewed twice a week, was supplied on part of the runway in Lots 11 and 12.

When the green clover was fed in a mixture with the basal ration, the mixture was made up fresh every 2 or 3 days and kept in a cooler at approximately 35°F. The fresh red clover used was of a second growth crop. It was cut in a small clover cutter, weighed out in exact quantities, and thoroughly mixed with the basal ration in a power meat grinder—thus rendering a thoroughly mixed mash.

The entire fourteen lots, except Lot 10 *a*, which received in addition to the ration a daily exposure (except Sundays) to direct sunlight after the 6th week, were allowed to remain on their respective rations and management for 12 weeks, when part of the surviving birds were killed for bone analysis (Table IV). Where bone analyses were desired the two tibias were dissected out, cleaned from adhering tissue, dried for 3 days at 105°C., then crushed between the jaws of a large pair of pliers, and quantitatively extracted with alcohol and ether for 18 hours, respectively. Subsequently, they were dried for 3 hours and then ashed in an electric muffle furnace. The percentage of ash (Table IV) is expressed on the alcohol-ether-extracted basis.

RESULTS AND DISCUSSION.

The protocols of this experiment as summarized in Tables I and II demonstrate rather conclusively that green clover will not prevent leg weakness even though fed to approximately 18 per cent of the ration (3:3 ratio)—calculated on the basis of the

dry weight of the clover. In every instance where green plant tissue was fed indoors, regardless of the percentage of intake, a nutritional disorder, characterized by a straddling and awkward

TABLE I.

Showing the Records of the Chicks Receiving the Basal Ration plus Various Amounts of Green Clover and $\frac{1}{2}$ Hour Exposure to (Direct) Sunlight.

Age.	Lot 1. Basal 3 + green clover 1.				Lot 2. Basal 3 + green clover 2.				Lot 3. Basal 3 + green clover 3.			
	Weight.	No. surviving.	No. with leg weakness.	Percentage of leg weakness.	Weight.	No. surviving.	No. with leg weakness.	Percentage of leg weakness.	Weight.	No. surviving.	No. with leg weakness.	Percentage of leg weakness.
<i>wks.</i>	<i>gm.</i>				<i>gm.</i>				<i>gm.</i>			
0	33.0	12	0	0	33.0	12	0	0	33.0	12	0	0
2	79.2	12	0	0	70.4	11	0	0	73.3	12	0	0
4	113.2	12	0	0	106.5	10	0	0	123.2	11	0	0
6	149.5	11	5	45.4	143.3	9	0	0	159.4	8	0	0
8	189.1	6	5	83.3	183.7	4	2	50.0	224.1	6	2	33.3
10	256.6	3	3	100.0	225.0	4	4	100.0	269.1	6	6	100.0
12	296.6	3	3	100.0	238.7	4	4	100.0	280.0	4	4	100.0

Age.	Lot 4. Basal 3 + green clover 1 + sunlight $\frac{1}{2}$ hr.				Lot 6. Basal 3 + green clover 2 + sunlight $\frac{1}{2}$ hr.				Lot 8. Basal 3 + green clover 3 + sunlight $\frac{1}{2}$ hr.			
	Weight.	No. surviving.	No. with leg weakness.	Percentage of leg weakness.	Weight.	No. surviving.	No. with leg weakness.	Percentage of leg weakness.	Weight.	No. surviving.	No. with leg weakness.	Percentage of leg weakness.
<i>wks.</i>	<i>gm.</i>				<i>gm.</i>				<i>gm.</i>			
0	33.0	12	0	0	33.0	12	0	0	33.0	12	0	0
2	77.5	12	0	0	87.7	11	0	0	81.6	12	0	0
4	146.3	11	0	0	124.5	10	0	0	133.2	11	0	0
6	202.2	9	0	0	180.0	9	0	0	188.6	11	0	0
8	306.6	9	0	0	283.7	8	0	0	277.5	10	0	0
10	472.2	9	0	0	460.6	8	0	0	423.0	10	0	0
12	628.3	9	0	0	617.5	8	0	0	603.0	10	0	0

gait, frequent squatting, and roughened condition of the feathers, developed, unless direct sunlight was furnished as an additional supplement. It appears that upon increasing the amount of green clover in the ration of a chick (Table I), the onset of leg weakness

is somewhat delayed. In Lot 1, 45 per cent of the birds gave unmistakable signs of leg weakness at the end of the 6th week. Upon increasing the ratio of green plant tissue to the basal ration (Lot 2), only 50 per cent of the birds gave evidence of the nutritional disorder at the end of the 8th week. Again, upon increasing the green feed to a 3:3 ratio, only 33 per cent of the birds gave evidence of leg weakness at the end of the 8th week. However, by the 10th week, all the birds not receiving direct sunlight exhibited the peculiar straddling gait. Thus it would appear that the green plant tissue furnished something that caused the delay of rachitic symptoms in proportion to increased intake of green clover. We are inclined to believe that this delayed onset of nutritional disturbance was due to the increased antirachitic vitamin intake; although not enough of this accessory factor was present to afford complete protection.

Regardless of the validity of our belief, green plant tissue unquestionably supplies something which prolongs life and stimulates growth. On the basal ration of white corn, middlings, casein, and salt mixture, chicks of the same nutritional history will attain an average weight of 85 to 90 gm. at the end of the 4th week when they rapidly start to fail, and none ordinarily survive the 6th week. By supplying green plant tissue (green clover) we not only prolong life beyond the 6th week but also obtain increased growth to the extent of 15 to 40 gm. per chick. This prolongation of life and increased growth we believe due in part to the increased amounts of the fat-soluble A vitamin, in which green plant tissue abounds, and also to some extent to increased intake of the antirachitic factor.

No success in the rearing of chicks is attained unless the antirachitic factor or its equivalent in the form of ultra-violet light is supplied in sufficient quantities. Tables I and II clearly demonstrate the beneficial effects of $\frac{1}{2}$ hour exposures to direct sunlight. In every instance the birds responded to the light treatment. Not only did they make more than twice the growth of the unexposed, but the malady of leg weakness was prevented and also cured with the production of an apparently normal chick whose signs of behavior were the exact antithesis of the unexposed. See Figs. 1 to 4.

The results of Lots 5, 7, and 9, receiving the same ration as

Lots 4, 6, and 8, respectively, but 1 hour exposure to sunlight instead of $\frac{1}{2}$ hour, are not represented because of lack of space. Suffice it to state that they were similar in growth and general behavior to those receiving $\frac{1}{2}$ hour of sunlight as borne out in the bone analysis (Table IV).

TABLE II.
Basal Ration plus Green Clover ad Libitum (Indoors).

Bird No.	0 wks.*	2 wks.	4 wks.	6 wks.	8 wks.	10 wks.	12 wks.
Lot 10 b.							
120	33	55	95	135	185	300	475†
128	33	90	140	165†	Dead.		
111	33	80	140	170	195†	210†	Dead.
103	33	70	130	190†	225†	225†	250†
188	33	80	130	150	175†	175†	185†
165	33	65	105	150	125†	Dead.	
123	33	65	115	140†	Dead.		
171	33	70	115	185	250†	"	
106	33	75	145	185	250	300†	350†
209	33	70	110	125†	Dead.		
Lot 10 a. $\frac{1}{2}$ hr. direct sunlight after 6th wk.							
189	33	70	125	175†	Dead.		
192	33	55	80	125†	160	140†	Dead.
144	33	60	85	125†	200	280	375
125	33	65	110	125	Dead.		
126	33	60	90	135†	200	275	445
199	33	50	100	115†	105†	120	200
194	33	55	70	80†	150	205	315
164	33	60	80	105	125	160	230
159	33	105	185	180†	360	625	945
295	33	85	165	220†	345	525	755

* Initial weights were averaged, all subsequent weights were individual.

† Exhibited signs of leg weakness.

‡ Sick, although no signs of leg weakness.

The remarkable stimulating effect of sunlight is further shown in Table III. Lot 13, receiving the basal ration which leads to leg weakness and failure indoors between the 4th and 6th weeks, shows without doubt the antirachitic and growth-promoting properties of direct sunlight (Fig. 5). Likewise, Lot 12 substantiates the



FIG. 1. The effect of $\frac{1}{2}$ hour exposure to direct sunlight after 6 weeks on a ration of (white corn, 2, wheat middlings, 1) 80, casein 16, salt mixture 4, plus chopped green clover *ad libitum* (left) in contrast to chick on the right which did not receive exposure to sunlight and exhibited signs of leg weakness (abnormal specimen). Weights 380 and 265 gm., respectively, at 11 weeks of age.



FIG. 2. The effect of feeding a ration of 3 parts of basal plus 1 part of green clover (by weight), with and without exposure to direct sunlight for $\frac{1}{2}$ hour daily (except Sundays). Cockerel on right (unexposed) suffering from leg weakness in contrast to cockerel on left (exposed) representing an apparently normal specimen. Weights 650 and 305 gm., respectively, at 11 weeks of age. Cockerel on left was free from leg weakness although it was in a squatting position at time of photographing.



FIG. 3. A contrast between the effect of green clover and direct sunlight. Both chicks received a ration of 3 parts of basal plus 2 parts of green clover (by weight). In addition the chick on the left received $\frac{1}{2}$ hour exposure to direct sunlight daily (except Sundays). Chick on right suffering from leg weakness; on left an apparently normal specimen (no leg weakness); weights 585 and 210 gm., respectively, at 11 weeks of age.



FIG. 4. A further contrast between the effect of green clover and direct sunlight. Both chicks received a ration of 3 parts of basal plus 3 parts of green clover (by weight). In addition the chick on the left (an apparently normal specimen) received $\frac{1}{2}$ hour exposure to direct sunlight daily (except Sundays). Chick on right (abnormal specimen) suffering from leg weakness. Weights 550 and 250 gm., respectively, at 11 weeks of age. Bird on left did not show signs of leg weakness although shown in a squatting position.

previous assertion that sunlight is an effective agent in preventing leg weakness. Apparently, soil which would furnish a great deal of extraneous material in the form of organic matter, minerals, etc. exhibited no marked improvement in nutrition aside from possibly lowering the rate of mortality. The beneficial effect of green feed, however, is rather outstanding. In both instances (Lots 13 and 14) where green clover was fed, there was a noticeable

TABLE III.

Showing the Weight and Mortality of the Chicks Having Access to an Outdoor Runway.

Age.	Lot 11. Sunlight, soil, and green clover <i>ad libitum</i> .		Lot 12. Sunlight and soil <i>ad libitum</i> .		Lot 13. Sunlight <i>ad libitum</i> .		Lot 14. Sunlight and green clover <i>ad libitum</i> .	
	Weight.	No. surviving.	Weight.	No. surviving.	Weight.	No. surviving.	Weight.	No. surviving.
<i>wks.</i>	<i>gm.</i>		<i>gm.</i>		<i>gm.</i>		<i>gm.</i>	
0	33.0	28	33.0	28	33.0	28	33.0	28
1	50.3	28	46.1	28	50.3	28	48.9	28
3	84.2	26	79.6	23	75.5	23	75.0	24
5	182.6	25	166.2	19	156.8	17	159.0	21
7*	366.3	25	275.6	18	266.7	15	310.1	19
9	458.1	25	397.5	18	382.2	15	445.8	19
12	777.3	25	691.5	18	716.3	15	764.8	19
Average on ♂ basis..	815.8		724.4		749.7		796.5	

* After the 7th week the cockerel and pullet weights were averaged separately and the two averages combined for a general average, which appears in the table.

We do not believe that the apparently high mortality in several lots was solely due to the ration. On the contrary, we contribute it jointly to management and ration, the ration being low in the fat-soluble A vitamin and the antirachitic factor.

increase in the weight of the birds, ranging approximately from 70 to 90 gm. Although all the birds in the outdoor lots were of a thrifty and normal appearance—it appeared to the authors that Lots 13 and 14, receiving green plant tissue, were the more choice birds in that they appeared more active and exhibited more bodily vigor.

The ash analysis of the tibias (Table IV), from representative chicks of each lot, is in agreement with the general nutritional

behavior of the chicks; the percentage of ash being low wherever the birds were deprived of direct sunlight regardless of the other supplements. In general the exposed birds showed an ash content from 13 to 15 per cent higher than the unexposed—thus conclusively showing the calcifying properties of direct sunlight.

The general practice of the poultryman to allow his chicks to run out of doors where they have access to sunlight, green plant tissue, and soil is well founded. However, from the results of this investigation we cannot subscribe to the general belief that green plant tissue will prevent the malady of leg weakness. On

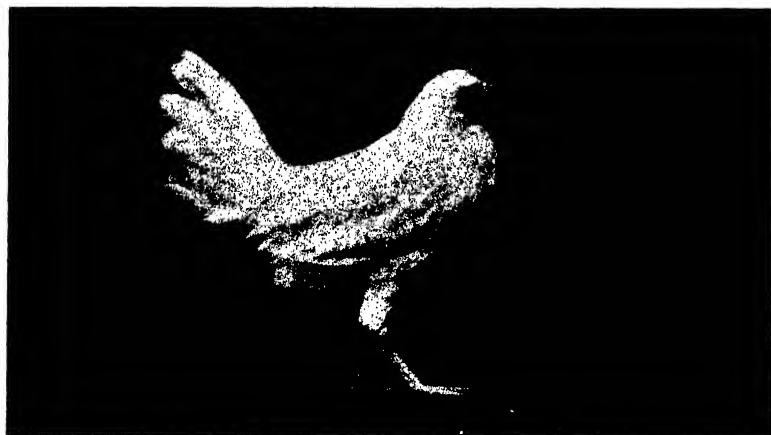


FIG. 5. The effect of unlimited radiant energy (sunlight) and a rachitic ration. Fed basal ration of (white corn, 2, wheat middlings, 1) 80; casein 16; salt mixture 4, in addition to an outdoor, elevated, runway. Weight 650 gm. at 11 weeks of age. Apparently a normal specimen.

the contrary, our results are indicative that the chick will not eat enough green clover of its own accord to prevent leg weakness, providing a ration low in the antirachitic factor is fed. Further we are doubtful whether a chick could ingest enough green clover, without other conflicting factors entering in, to protect itself from leg weakness. However, we do not wish to infer that green plant tissue does not carry the antirachitic factor and that it is of no value in the dietary of a chick. On the contrary, we recognize the occurrence of antirachitic properties in green plant tissue; but believe that green clover, as used in this experiment, does not

TABLE IV.
Showing the Percentage of Ash in Tibias (Expressed on Alcohol-Ether-Extracted Basis).

Basal 3 + clover 1.						Basal 3 + clover 2.						Basal + clover ad libitum.											
Lot 1.			Lot 4. ‡ hr. sunlight.			Lot 5. 1 hr. sunlight.			Lot 2.			Lot 6. ‡ hr. sunlight.			Lot 7. 1 hr. sunlight.			Lot 10 b.			Lot 10 a. ‡ hr. sunlight.*		
No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent
387	39.58		445	54.51		457	52.00		400	39.96		446	54.28		94	56.04		120	43.30		126	54.20	
390	38.31		500	56.58		486	51.84		352	40.91		496	56.42		450	51.26		103	39.46		194	55.11	
368	41.97		424	52.14		422	52.08		355	40.22		480	55.22		444	55.20		188	40.01		164	55.16	
			493	51.92		454	56.85		315	41.49		469	52.42		152	57.15		106	41.39		144	53.39	
			481	53.28		406	52.90					455	57.08		490	56.97					295	56.43	
Average..	39.95			53.68			53.13			40.64			55.08			55.32			41.04			54.86	

Basal 3 + clover 3.						Basal + outdoor runway (sunlight) plus																	
Lot 3.			Lot 8. ‡ hr. sunlight.			Lot 9. 1 hr. sunlight.			Lot 11. Clover and soil ad libitum.			Lot 12. Soil ad libitum.			Lot 13.			Lot 14. Clover ad libitum.					
No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent	No.	Ash.	per cent
443	40.85		17	57.24		576	57.86		907	56.30		930	58.41		927	52.35		929	53.54				
429	38.63		184	55.51		1651	56.81		985	55.42		933	58.27		926	56.67		917	56.79				
473	42.25		45	58.81		141	57.92		916	54.65		921	57.38		960	58.46		957	55.31				
484	42.73		124	57.05		1766	53.62		945	56.33		920	53.40		934	54.90		996	54.97				
			95	53.41		1772	56.40		928	53.82		964	53.25		923	56.45		997	58.01				
Average..	41.38			56.40			56.52			55.30			56.14			55.76			55.72				

* ‡ hour exposure to (direct) sunlight after 6 weeks on basal ration plus green clover ad libitum.

contain enough of this accessory factor, even at a level of approximately 18 per cent of the ration, calculated on the basis of the dry weight of the clover, to protect a chick from leg weakness.

Shipley, Kinney, and McCollum (8) have observed in the case of the rat that an ether extract of the clover plant fed on the basis of 250 gm. to a kilo of ration would produce healing of rachitic symptoms in a period of 7 days with complete healing in 33 days. Undoubtedly in the case of chicks more protection or a delayed onset of leg weakness would result if 25 per cent green clover, on the basis of the dry weight, were employed. We, however, question whether complete protection would result, as the indications are that the antirachitic requirements of a growing chick are greater than those of a rat.

There can be no question as to the beneficial effects of direct sunlight. Our results indicate that $\frac{1}{2}$ hour of direct sunlight is more effective in preventing leg weakness in chicks than green clover fed at a level of approximately 18 per cent, calculated on the basis of the dry weight of the clover. We, therefore, feel justified in assuming that direct sunlight is a better preventive of leg weakness than green clover and that the beneficial effect from turning chicks out of doors in this respect is not so much due to the ingestion of green plant tissue and other extraneous materials as it is to the effect of radiant energy (direct sunlight.)

SUMMARY.

Green (fresh) red clover fed to an approximate level of 18 per cent of the ration, calculated on the basis of the dry weight of the clover, did not protect the chick from leg weakness, although the feeding of green plant tissue prolonged life, increased growth, and delayed the onset of leg weakness.

$\frac{1}{2}$ hour of direct sunlight proved more beneficial in preventing leg weakness than 18 per cent of green clover, calculated on the basis of the dry weight of the clover.

Soil, apparently, did not greatly benefit the nutrition of the chick, when other nutrients were supplied, aside from possibly decreasing the rate of mortality.

Sunlight appeared not only to act as a calcifying agent of the skeletal tissue, thus preventing the occurrence of leg weakness, but also seemed to possess growth-promoting properties.

We believe the beneficial effects ordinarily ascribed to green plant tissue and extraneous materials ingested when chicks are out of doors on range are not as great as those of direct sunlight, so far as the antirachitic factor is concerned.

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NOTE ON THE CONCENTRATION OF THE ACETONE BODIES IN NORMAL BLOOD AND URINE.

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In the course of an experiment on the development of a mild degree of ketosis brought about by the ingestion of a diet low in carbohydrate, two specimens of blood were obtained from the subject under investigation. The first was drawn approximately at the beginning of the experiment, and the second after the diet had been fed for 3 days. In these determinations the concentrations of the acetone bodies were approximately the same in the blood and urine in the specimens obtained at the beginning of the experiment, while later the concentration was much higher in the urine than in the blood. It has seemed worth while to get together more data upon the relative concentrations of these compounds in normal blood and urine to serve as a basis for further studies upon the thresholds of the compounds when they are present in increased amounts.

Data from twenty-nine experiments upon twenty-four subjects are reported. In each case a specimen consisting of about 20 cc. of blood was drawn in a syringe, and discharged at once into a bottle containing potassium oxalate. The next specimen of urine voided was obtained, and the specimens of blood and urine were analyzed simultaneously by methods previously described (Hubbard, 1921, *a*; 1921, *b*). Occasionally, the filtrates, after the protein and interfering compounds had been precipitated, stood in stoppered containers on ice overnight before the analyses were made. As far as could be determined within the limits of accuracy of the methods used, this procedure had no effect upon the results. A modification of the Messinger (1888) titration employing hundredth normal solutions of iodine and thiosulfate

solutions was used in the final analysis. The limitations of the technique have been discussed in the articles to which reference has already been made. An attempt was made to control the most important source of variations—differences in the amount of interfering volatile compounds introduced from the air of the laboratory during the procedure—by carrying through analyses of both blood and urine simultaneously. Frequent determinations of the titration value given by the reagents were made during the course of the experiments, and the averages of these values have been subtracted from the determinations. In a number of instances the values found from the specimens were within the range of values found for the controls. The limit of accuracy of the methods was probably about 0.2 mg. per 100 cc. of blood or urine; it was almost certainly better than 0.5 mg. per 100 cc.

In Table I all of the results except the first four are given. These have been omitted because the precipitation of protein and the determination of acetone in blood did not run smoothly at first because of errors in the standardization of the reagents, and the results were somewhat, but not markedly, more irregular than were those obtained later. Duplicate determinations upon all of the subjects studied in this fore period were obtained, and results upon them included in the table.

The table shows that the amounts of the acetone bodies found in normal blood and urine were very small—about 0.0 to 0.3 mg. per 100 cc. These figures approximately agree with results upon normal subjects usually, but not universally, found by different methods in different laboratories. These results lie within the limits of accuracy of the method used, and no great emphasis can be placed upon differences in the values found in the different subjects, or upon the relative amounts found in the blood and the urine from the same subject. The amounts of acetone from both fractions determined were approximately the same in both blood and urine. This is particularly noticeable in the case of the studies of acetone from preformed acetone plus acetoacetic acid. The averages of all values of this fraction were practically identical in blood and urine, and in only four instances were the differences between the acetone content of the two fractions large enough to be of any significance. This fact seems suggestive. Widmark (1920) has shown that acetone is indefinitely diffusible

TABLE I.

Date.	Subject.		Blood determinations.			Urine determinations.		
	No.	Sex.	Filtrate equiva- lent to.	Acetone bodies in 100 cc.		Filtrate equiva- lent to.	Acetone bodies in 100 cc.	
				A*	B*		A*	B*
1924			cc.	mg.	mg.	cc.	mg.	mg.
July 18	1	M.	5.0	0.6	2.6	7.5	0.4	1.4
" 19	2	"	5.0	0.0	0.0	7.5	0.0	0.5
" 19	3	"	3.5	0.0	0.0	7.5	0.0	0.5
" 19	1	"	7.5	0.2	0.0	7.5	0.0	0.0
" 22	4	"	7.5	0.0	0.0	7.5	0.0	0.0
" 30	1	"	7.5	0.0	1.3	7.5	1.2	1.9
" 31	5	F.	7.5	0.1	0.3	7.5	0.0	0.0
" 31	4	M.	7.5	0.0	1.3	7.5	0.0	0.3
Aug. 1	6	F.	5.0	0.2	0.0	7.5	0.0	0.2
" 1	7	M.	5.0	0.2	0.0	7.5	0.0	0.2
" 3	8	"	4.5	0.9	0.0	7.5	0.0	0.0
" 3	1	"	7.5	0.0	0.0	7.5	0.0	0.2
" 5	9	"	7.5	0.0	0.0	7.5	0.0	1.1
" 6	1	"	7.5	0.1	0.0	7.5	0.0	0.3
" 7	10	"	5.0	0.0	1.7	7.5	0.0	1.9
" 8	11	"	6.8	0.0	0.0	7.5	0.0	1.1
" 9	12	"	7.5	0.0	0.0	7.5	0.0	0.0
" 9	13	"	7.5	0.0	0.1	7.5	0.4	0.0
" 12	14	"	7.5	0.1	0.3	7.5	0.0	0.0
" 12	15	"	6.5	0.0	0.0	7.5	0.4	0.0
" 15	16	"	7.5	0.3	0.0	7.5	1.2	2.6
" 15	17	"	7.5	0.1	0.9	7.5	0.0	0.9
" 16	18	F.	7.5	0.1	0.0	7.5	0.9	0.3
" 18	19	"	7.5	0.3	0.0	7.5	0.1	0.0
" 26	20	"	7.5	0.3	0.0	7.5	0.4	0.5
" 26	21	"	7.5	0.1	0.0	7.5	0.0	0.0
" 28	22	"	7.5	0.1	0.0	7.5	0.1	0.0
" 28	23	M.	7.5	0.3	0.0	7.5	0.1	0.0
" 29	24	F.	7.5	0.0	0.0	7.5	0.3	0.2
Average.....				0.13	0.30		0.19	0.46

* Under "A" the amounts of acetone from preformed acetone plus acetoacetic acid, and under "B" those from β -hydroxybutyric acid are given.

in the human body, and it has been pointed out that one of the products of carbohydrate disintegration in the intestine may be acetone (Alvarez, 1924). It is impossible to conclude that the acetone found in this fraction in the blood is present as acetone, for, as already pointed out, the amounts found are practically inside of the limit of accuracy of the method; it is reasonable, however, to conclude that the amounts of acetoacetic acid in normal blood must be very small indeed. The results of the determination of β -hydroxybutyric acid in normal urine are more irregular, and there is more difference in the average amounts in blood and urine than is the case of acetone from the other fraction. As has been pointed out in earlier work (Hubbard, 1921, a), there is no proof that the compound in normal blood which gives rise to acetone or a compound related to acetone on oxidation with sulfuric acid and potassium dichromate, and which is reported as β -hydroxybutyric acid, actually is that acid. If the acetone bodies which we conceive as intermediate products of metabolism are present in normal blood, they are present only in very small amounts.

Samples of blood and urine obtained simultaneously from normal subjects were analyzed for their content of the acetone bodies by methods which have an accuracy of about 0.2 mg. per 100 cc. Acetone from preformed acetone plus acetoacetic acid and from β -hydroxybutyric acid was found in most of the samples of blood and urine in concentrations of about 0.0 to 0.3 mg. per 100 cc. The results upon the blood and urine in a large majority of the cases agreed within the limit of accuracy of the method.

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THE EXTRACTION OF A PARATHYROID HORMONE WHICH WILL PREVENT OR CONTROL PARATHYROID TETANY AND WHICH REGULATES THE LEVEL OF BLOOD CALCIUM.*

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INTRODUCTION.

The communication represents the preliminary results of a direct attack upon the problem of the suggested hormonal function of the parathyroid glands. The literature on the parathyroid glands is most voluminous, and as excellent reviews have recently been published by Boothby (1921) (1) and Simpson (1922) (2), no attempt at a detailed review of the literature on this subject will be made at this juncture.

Function of the Parathyroids.

It is generally agreed that the parathyroid glands are vital organs and that complete extirpation of these structures results in death in a few days, death being preceded by a definite clinical syndrome—tetania parathyreopriva. Various theories have been advanced to account for the fatal issue following complete parathyroidectomy. MacCallum (1911 and 1912) (3) and MacCallum and Vogel (1913) (4) by cross circulation experiments showed that the hyperexcitability of the nervous system is peripheral and that it is dependent on some change in the character of the blood. They showed that if the blood from an animal in tetany were perfused through a normal leg, the excitability of the nerves rose to

* It was the original intention of the writer to adopt for the specific name of the parathyroid hormone herein described, the term "parathyrin" which had previously been suggested by Sharpey-Schäfer for an hypothetical autocoid elaborated by the parathyroid gland. Since the manuscript of this communication has been submitted for publication, it has been learned that a proprietary firm has applied for a copyright on this word. Pending the action of the United States copyright office, a specific name is for the time being not definitely chosen.

a characteristic high level and that the addition of parathyroid extract had little or no effect in lowering this excitability. Koch (1912 and 1913) (5) found methyl guanidine in the urine of parathyroidectomized dogs. Paton, Findlay, and Burns (1915) (6) showed that the injection of guanidine and methyl guanidine apparently produces all of the characteristic symptoms of tetany in rats. Paton and Findlay with Watson, Burns, Sharpe, and Wishart later (1916) (7) presented a series of papers, which aimed to show the etiology of tetany and its relationship to guanidine and methyl guanidine intoxication. They concluded that the symptoms of tetany were due to some change in the nerve cells of the cord, and that the increased excitability of the neuromyon is caused by an action on the nerve endings. As a result of an extensive comparison between the symptomatology of idiopathic tetany, postoperative tetany in man, tetania parathyreopriva, and guanidine poisoning, they concluded that all these conditions are of the same nature and that the first three are due to the development of guanidine in the body as the result of some interference with the action of the parathyroid glands which normally control guanidine metabolism.

Parhon and Urechie (1907) (8) and MacCallum and Voegtlin (1908) (9) discovered that calcium has a palliative effect in parathyroid tetany. MacCallum and Vogel (1913) (4) showed by direct analysis that the blood of an animal in tetany is low in calcium content. Howland and Marriott (1918) (10) found that the calcium of the blood serum of normal persons varied from 9.2 to 11.3 mg. per 100 cc. of serum, whereas the calcium of serum of patients in tetany of idiopathic origin was reduced by 3.5 to 7.3 mg. or an average of 5.6 mg.

Dragstedt (1922) (11) and Dragstedt and Peacock (1923) (12) found that completely parathyroidectomized animals could be kept alive indefinitely if kept on a special carbohydrate diet which consisted in the main of white bread, lactose, and milk *ad libitum*.

Dragstedt, Phillips, and Sudan (1923) (13) stated: "The experimental evidence seems most in harmony with the theory that the parathyroid glands form a part of the detoxicating mechanism of the body."

Luckhardt and Rosenbloom (1921) (14) stated that completely parathyroidectomized animals could be cured of all symptoms of parathyroid tetany by the intravenous injection of Ringer's solution. Luckhardt and Goldberg (1923) (15) found that completely parathyroidectomized dogs could be kept alive, without showing tetany, on a meat diet, by means of the daily oral administration of calcium lactate in 1.5 gm. per kilo doses.

The recent studies of Salvesen (1923) (16) show that parathyroid tetany is due to low blood calcium and that the function of the parathyroid glands is to regulate the level of blood calcium.

Greenwald (1924) (17) was unable to demonstrate a toxin in the blood of parathyroidectomized animals in a state of tetany. This investigator elsewhere states (1924) (18): "There are two, and only two, well authenticated metabolic changes after parathyroidectomy. One is the lowered

calcium content of the serum or plasma and the other is the diminished excretion of phosphorus in the urine. These must be regarded as being intimately connected with the sequence of symptoms observed. No theory of the causation of tetany can be considered adequate if it fails to take both of these into consideration." His own experiments he states: "seem to offer good reason for refusing to accept, without further evidence, the idea that tetany is due to guanidine intoxication."

To sum up the theories of parathyroid function which have been advanced, the balance, in the opinion of the writer, swings in favor of a hormonal function having to do normally with the regulation of calcium metabolism through a specific control of blood calcium concentration.

Parathyroid Extracts.

Numerous attempts have been made in the past to prepare parathyroid extracts with a view to their use in combating tetany. Some investigators have reported positive findings following the use of certain parathyroid preparations. Berkeley and Beebe (1909) (19) prepared an extract from beef parathyroids from which they separated a nucleoprotein that appeared to them to have curative properties when administered to parathyroidectomized animals. Many other investigators later failed to find curative properties in parathyroid preparations. Sharpey-Schäfer (1924) (20) states:¹ "It is now generally agreed that the parathyroid secretion produces no acute effect when injected into the blood." He is of the opinion,² however, that the parathyroids pass into the blood an autocoid which assists in promoting protein metabolism and enables intermediate products such as guanidine to be further metabolized.

Recently, Berman (1924) (21) claimed to have a preparation of beef parathyroid glands which definitely raises the calcium content of the blood when dissolved in Ringer's solution and injected into the circulation. He states that this is additional evidence in favor of the view that the parathyroid glands secrete a hormone that influences the calcium content of the blood.

Parathyroid Grafts.

The most conclusive evidence hitherto brought forward that the parathyroids furnish an essential internal secretion has been furnished by grafting experiments. Postoperative tetany in man has frequently been relieved by a parathyroid graft. Borchers (1919) (22) stated that a single large parathyroid is adequate to obviate symptoms in the human subject. Biedl (1916) (23) transplanted the parathyroids of a dog into

¹ Sharpey-Schäfer (20), p. 85.

² Sharpey-Schäfer (20), p. 84.

the spleen of the same animal and subsequently removed the thyroids. No symptoms of tetany showed themselves during some months after the operation. When the spleen was later removed, however, fatal tetany supervened within 24 hours.

The Present Work.

With a conviction that the parathyroid gland contains a hormone which, if obtained in potent form, could be used successfully in replacement therapy, an attempt was made to extract it. We had already satisfied ourselves that the maintenance of a normal calcium content of the blood was the object to be attained since, in confirmation of Luckhardt and Goldberg (15) and of Salvesen (16), we had shown that parathyroidectomized dogs could be kept in a normal condition on a meat diet either by the oral administration of massive doses of calcium lactate or by the intravenous administration of relatively small amounts of calcium chloride. When we endeavored to control tetany in parathyroidectomized dogs with parathyroid extract alone, we obtained our first conclusive results with an extract made by acid hydrolysis of the parathyroid glands of the ox. Once definitely positive results were obtained it was found that the potency of the extract could be greatly increased and that results could be readily duplicated. By the use of this extract we were able to control or prevent tetany in parathyroidectomized dogs. A point of great interest which appeared very early in the investigation was the close parallelism between the clinical condition of our animals and the calcium content of their blood. Coincident with the clinical improvement following the use of our extract the blood calcium level was raised or restored to normal. Later on in the investigation it developed that an overdose of the parathyroid preparation was a possibility and in such a case an abnormally high blood calcium content was found, and along with this blood picture there were associated typical clinical manifestations.

Methods.

Dogs were used as the experimental animals. Young animals were selected by preference when available on account of their greater susceptibility to early fatal tetany following thyroparathyroidectomy. The animals received no preliminary diet treatment prior to operation because this also renders them more susceptible to tetany. Stock animals were

fed kitchen scraps containing considerable meat. Other animals were used immediately after they were received and these had presumably been on a mixed diet. Morphine was administered 2 to 3 hours before operation. This had a twofold beneficial effect. The animal as a rule both vomits and defecates shortly after the administration of morphine and later takes an anesthetic well. Atropine was injected just prior to the induction of ether anesthesia. The operation was carried out according to the usual surgical technique. A complete thyroparathyroidectomy was done, care being taken to keep outside the capsule and to clear away deeply at each pole. Our experience has been that dogs without any preliminary dietary treatment subjected to this operation develop, as a rule, fatal tetany in from 24 to 48 hours. There are three dogs in the series herein reported in which definite tetany was not manifested. They were all old animals and all had received prophylactic treatment with potent parathyroid preparations prior to the "proving up" period. In the early successful experiments the animals were kept on a bread and water diet, in some instances distilled water only being given. Later on, operated animals were placed on a meat diet at once.

Preparation of Parathyroid Extracts.

Parathyroid glands were removed from oxen at the time of slaughtering. Adherent fat and connective tissue were removed and the glands were chilled and frozen at once. They were transported to the laboratory packed in ice and were at once placed in an ice box at -4°C . When required for use a few glands were placed in a large Pyrex test-tube and covered with an equal volume of 5 per cent HCl. The test-tube was placed in a boiling water bath for 1 hour, the glands being broken up after a few minutes heating by means of a glass rod.

After the necessary period of digestion had elapsed the fat which had separated out as an oily layer on the surface of the extract was removed mechanically. The extract was chilled at once to room temperature and made alkaline to pH 8 by NaOH. HCl was then added slowly till a maximal precipitation of protein and protein derivatives occurred. The precipitate was removed at this point either by the centrifuge or by filtering. The precipitate was redissolved in weak alkali, and a second isoelectric precipitation carried out. The filtrates or, if the centrifuge was used, the supernatant fluids, were combined, and this preparation represented an aqueous solution of the active principle. It was kept in the ice chest until required for use. This form of preparation of the parathyroid hormone seemed to possess great potency. It could be administered in any one of three ways; namely, by stomach, by subcutaneous injection, or by intravenous injection. If either of the latter methods were employed the extract could be sterilized by heating in a boiling water bath. The hormone itself appears to be a fairly simple organic substance, and the highly potent aqueous preparation above described has been treated with different reagents to determine its solubilities. This

part of the work is only now in the preliminary stages, since we have used for the most part a known potent aqueous extract in most of our animal experiments reported in this communication. Now that the physiological significance of the hormone is, we feel, definitely established, the problem of purification is being studied more elaborately.³

The extract used in the early experiments was prepared by making the defatted acid hydrolysate of the glands alkaline to pH 8. If the intravenous route was used for administration, the alkaline extract was filtered, whereas if the oral route was used, the unfiltered extract was given by stomach tube. The extracts first used were made definitely alkaline deliberately because of the possible transient beneficial effect that acid might elicit. This alkaline extract will be referred to as crude extract, whereas the isoelectric filtrate used later will be so designated. The volume of the extracts was adjusted so that 1, 2, or 3 cc. were the equivalent of one ox gland.

Blood samples were taken from a leg vein, and the calcium content of the serum determined by the Tisdall-Kramer method, with certain slight modifications which make for greater accuracy.

RESULTS.

The detailed protocols of the experiments are shown in tabular form in Table I. The effect of the extract upon blood calcium values is shown in Table II.

The restoration of a parathyroidectomized dog in a state of tetany to normal by the use of the parathyroid extract is shown in Figs. 1 and 2.

DISCUSSION.

Although the work on this problem is as yet in the preliminary stages, the results shown above are such as to leave no doubt whatsoever that a potent extract of the parathyroid glands has been obtained. The following points are submitted in substantiation of this statement.

³ Since going to press great progress has been made towards purification and concentration of the active principle contained in the extracts, the use of which is herein described. While the earlier extracts could be given by subcutaneous or intravenous injection without any local reaction being elicited, it was recognized that they were nevertheless relatively crude and also that they had the disadvantage of losing potency in the course of a few days following their preparation. The extract which is now being used is a much more pure and at the same time relatively stable product. Details of the preparation and properties of the further purified hormone will be published forthwith.



FIG. 1. Dog 32. 59 days after thyroparathyroidectomy. In a state of tetany.



FIG. 2. Dog 32. 59 days after thyroparathyroidectomy. Complete recovery 3 hours after subcutaneous injection of 3 cc. of parathyroid extract.

TABLE I.

Animal.	Date.	Time.	Blood calcium. mg. per 100 cc. serum	Extract given.	Remarks.
Dog 26. ♀ Cocker spaniel. 10 kilos.	1924				
	Oct. 7	3.00 p.m.			Thyroparathyroidectomy.
	" 8	5.20 "			Tetany. (Panting; tremors.)
		5.30 "			Intravenous.
		6.15 "			Breathing normal.
	" 9	8.30 a.m.			Normal.
		9.00 "			Spasmodic heavy respirations.
		1.00 p.m.			Respirations rapid; tremors in muscles.
		5.30 "		3 "	By stomach tube.
	" 10	8.30 a.m.			Animal weak but otherwise normal.
		3.30 p.m.			Definite tetany.
		3.30 "		3 "	By stomach tube. Vomited at once.
		4.05 "			Strychnine-like convulsions.
	" 11	5.40 "		2 "	By stomach tube.
Dog 27. ♂ Collie. 22 kilos.		8.30 a.m.			Animal shows no signs of tetany.
					Respirations are normal.
	" 12	1.00 p.m.		2 "	By stomach tube.
	" 13	5.30 "		2 "	Animal normal all day.
	" 14				Refused to eat. No signs of tetany.
		8.30 a.m.			Found dead.
	Oct. 14	4.00 p.m.			Thyroparathyroidectomy.
	" 15	3.00 "			Panting.
		6.00 "			Definite tetany. Hyperpnea; tremors and spastic gait.

	Oct. 16	6.00 p.m. 8.15 a.m.		0.1 gm. desiccated gland.	Extract made as before. Given by stomach tube. Found dead.
Dog 28. ♂ Mongrel. 17 kilos.	Oct. 14 " 15 " 16	5.00 p.m. 6.00 " 6.30 " 8.30 a.m.		2 glands crude.	Thyroparathyroidectomy. Onset of tetany. By stomach tube. Found dead.
Dog 29. ♂ Collie. 28.5 kilos.	Oct. 10 " 11 " 12 " 13 to 21	5.00 p.m. 1.00 " 5.30 "		3 glands crude. 2 "	Thyroparathyroidectomy. By stomach tube. Animal normal. " " " " No extract. 500 gm. meat daily. No tetany. Animal used for other purposes, Oct. 21.
Dog 30. ♂ Mongrel. 17 kilos.	Oct. 18 " 19 " 20	11.30 a.m. 2.30 p.m. 8.30 a.m.		2 glands crude.	Thyroparathyroidectomy. Intravenous. Animal shows no signs of tetany. Found dead.
Dog 31. ♂ Retriever. 22 kilos.	Oct. 21 " 22 " 23 " 24 " 25 " 26 " 27 " 28 " 29	10.30 a.m. 5.30 p.m. 5.30 " 7.00 " 5.00 " 5.00 " 4.00 "	10.5	2 glands crude. 2 " 2 " 2 " 2 " 2 "	Thyroparathyroidectomy. Intravenous. " Animal normal. " " " " " " Animal normal. " " Intravenous. Animal normal.

TABLE I—Continued.

Animal	Date	Time	Blood cal- cium. <i>mg. per 100 cc. serum</i>	Extract given.	Remarks.
Dog 31—Cont.	1934				
	Oct. 30	10.00 a.m.		3 glands 82 per cent alcohol.	500 gm. raw meat. Animal normal.
	" 31	6.00 p.m.			By stomach tube. " "
		9.30 a.m.			700 gm. raw meat. " "
		6.30 p.m.	7.7	3 " isoelectric filtrate.	By stomach tube. Slight muscle tremors noted.
	Nov. 1	6.30 "		3 " crude.	By stomach tube.
	" 2				Normal.
	" 3	9.30 "	6.9	2.5 " "	By stomach tube. 800 gm. meat.
	" 4	9.30 a.m.	9.2		Normal. 800 gm. meat.
		10.30 p.m.	7.1	15 cc. dialysate (unknown strength).	By stomach tube.
	" 5	10.00 a.m.	7.5	4 glands isoelectric filtrate.	Normal. 800 gm. meat.
	" 6 to 8	3.45 p.m.	7.5	3.5 " crude.	Intravenous.
	" 9 to 11			No extract.	By stomach tube, morning and evening. 800 gm. meat daily.
	" 11	10.00 a.m.	9.02		Normal. 800 gm. meat daily.
Dog 32. ♀ Cocker spaniel pup. 3.3 kilos.	Oct. 20	11.00 a.m.			Returned to kennel. No symptoms to date, Nov. 28.
	" 23	12.00 m.			Thyroparathyroidectomy.
		12.35 p.m.	7.5	2 glands crude.	Tetany.
		2.30 "			Intravenous.
		5.30 "	10.0	0.5 gland	Normal.
	" 24	7.00 "		2 glands	Intravenous.
	" 25	5.00 "		2 "	" Normal.

Oct. 26	5.00 p.m.		2 glands crude.	Intravenous. Normal.
" 27	5.00 "		2 " "	" "
" 28	5.00 "	6.8	2 " "	" "
" 29	6.00 "		2 " "	By stomach tube. Normal.
" 30	6.00 "		2 " "	" " "
" 31	6.30 "		2 " "	" " "
Nov. 1	6.30 "		2 " "	" " "
" 2			No extract.	Normal.
" 3	4.00 "			Onset of tetany. Tremors and yelping.
	4.30 "	5.5	2 cc. solution of precipitate with 92 per cent alcohol.	
" 4	10.00 "	6.6	2 glands crude.	By stomach tube. Animal quiet.
" 5	6.30 "		2 " "	" " " normal.
	10.00 a.m.		2 " "	" " " Muscle tremors noted.
" 6			2 " "	By stomach tube. 9 a.m. and 9.30 p.m. Normal.
" 7	6.00 p.m.		2 " "	By stomach tube. Normal.
" 8	1.00 "		2 " "	" " "
	12.00 m.		1 gland	" " "
" 9	10.00 a.m.		1 " "	" " "
	10.00 p.m.		1 " "	" " "
" 10	10.00 a.m.		1.5 glands	" " "
				Tetanic spasm when stomach tube was passed.
	7.00 p.m.		2 " "	Normal.
" 11	10.00 a.m.		2 " "	Leg muscles slightly spastic.
	7.00 p.m.		2.5 " "	" " "
				" " "

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium.	Extract given.	Remarks.
Dog 32—Cont.	1924		<i>ml. per 100 cc. serum</i>		
	Nov. 12	10 00 a.m.		2 glands crude by stomach tube.	Leg muscles slightly spastic.
	" 13	7 00 p.m.		2 " " "	" " "
	" 13	9 00 a.m.		2 " " "	" " "
	" 14	6 00 p.m.		2 " " "	Normal.
	" 14	11 00 a.m.		2 " " "	"
	" 15	7 00 p.m.		2.5 " " "	"
	" 15	9 00 a.m.		2.5 " " "	"
	" 16	6 00 p.m.		2.5 " " "	"
	" 16	10 00 a.m.		3 " " "	"
	" 16	10 30 p.m.		3 " isoelectric filtrate.	Subcutaneous. Stomach tube was passed and animal went into a tetanic spasm. Artificial respira- tion for 3 min.
	" 17	10 30 a.m.		1 gland " "	Subcutaneous. Normal.
	" 18	6 00 p.m.		2 glands " "	" "
	" 18	10 30 a.m.		1 gland " "	" "
	" 19	9 30 p.m.		1 " " "	" "
	" 19	1 00 "		2 glands " "	" " 100 gm. lean meat.
	" 20	6 30 "		2 " " "	Subcutaneous. Normal.
	" 20	9 00 a.m.		" " "	Animal drowsy.
	" 21	5 30 p.m.		2 " " "	Subcutaneous. Normal. 100 gm. lean meat.
	" 21	10 00 a.m.		" " "	100 gm. meat. Normal.

		6.00 p.m.		1 gland isoelectric filtrate.		Subcutaneous. 100 gm. meat. Normal.
Dog 33. ♂ Cocker spaniel pup. 4.8 kilos.	Nov. 22	6.00 "		1.5 glands "	"	"
	" 23	9.00 "		1.5 " "	"	"
	" 24	10 00 a.m.		3 " "	"	Subcutaneous. 100 gm. meat. Normal.
		4.30 p.m.	5.5		"	Animal in most violent tetany.
		5.30 "				" sitting up.
		8.00 "				Complete recovery. 100 gm. meat eaten during night.
	" 25	6.00 "		2 " "	"	Slightly spastic. 200 gm. meat during day.
	" 26	6.00 "		2 " "	"	Normal. 200 gm. meat during day.
	" 27	6.00 "		2 " "	"	" 200 " "
	" 28	6.00 "		2 " "	"	" 200 " "
	Oct. 24	4.00 p.m.		2 glands crude.		Thyroparathyroidectomy.
	" 25	1.00 "				Intravenous. Tetany; panting; muscle tremors.
	" 26	5.00 "		2 " "		Intravenous. Muscle tremors.
	" 27	9.00 a.m.	5.6	2 " "		" Tetany.
		10.00 "				No signs of tetany.
		12.00 m.	10.8			Normal.
		5.00 p.m.		2 " "		Intravenous.
	" 28	4.00 "	5.9	2 " "		" Tetany.
	" 29	6.00 "	6.9	3 " "		By stomach tube. No tetany.
	" 30	6.00 "		2 " "		" " Normal.

TABLE I—Continued.

Animal.	Date.	Time.	Blood calcium. mg. per 100 cc. serum	Extract given.	Remarks.
Dog 33—Cont.	1924				
	Oct. 31	6.30 p.m.		2 glands crude.	By stomach tube. Normal.
	Nov. 1	6.30 "		2 " "	" " "
	" 2			No extract given.	Normal.
	" 3	5.00 "	5.1	3 cc. = solution of precipitate from 92 per cent alcohol.	Intravenous. Tetany; tremors; irritable.
	" 4	10.30 "		2 glands crude.	By stomach tube.
		10.00 a.m.	5.1	2 " "	" " " No tetany but animal definitely groggy.
		6.30 p.m.		2 " "	By stomach tube.
		10.00 "		2 " "	" " " Spastic early in evening.
	" 5	10.00 a.m.		3 " "	By stomach tube.
		3.30 p.m.	4.2	3 " " isoelectric filtrate.	Intravenous. Tetany.
		11.00 "	5.2	2 " " crude.	By stomach tube. Some improvement. Muscles still slightly spastic.
	" 6	9.30 a.m.		2 " "	By stomach tube. Tremors.
		4.30 p.m.		2 " "	" " "
		9.30 "		2 " "	" " "
	" 7	10.00 a.m.		5 " "	" " " Spastic.
		6.00 p.m.		5 " "	" " " " During day there was considerable improvement.
	" 8	1.30 "		2 " "	Normal.
		12.00 m.		2 " "	" "

	Nov. 9 to 16		2 glands crude.	1 gland isoelectric filtrate.	Twice daily.	Normal throughout.
	" 17	10 30 a.m.	1 gland	"	Subcutaneous.	"
	" 18	6 00 p.m.	2 glands	"	"	"
	" 19	10 30 a.m.	2 "	"	"	"
	" 20	9 30 p.m.	2 "	"	"	"
	" 21	1 00 "	2 "	"	"	100 gm. lean meat.
	" 22	6 30 "	2 "	"	"	"
	" 23	5 30 "	2 "	"	"	100 gm. lean meat.
	" 24	10 00 a.m.	1 gland	"	100 gm. meat.	"
	" 25	6 00 p.m.	1.5 glands	"	100 " "	Normal.
	" 26	8 00 "	1.5 "	"	100 " "	"
	" 27	9 00 a.m.	0.5 gland	"	200 " "	"
	" 28	6 00 p.m.	7.6	"	200 " "	"
	" 29	7 00 p.m.		"	Subcutaneous. 200 gm. meat.	Animal somewhat weak.
	" 30	9 00 a.m.		"	Normal. 100 gm. meat.	"
	" 31	6 00 p.m.	1.5 glands	"	Subcutaneous. Normal.	100 gm. meat.
	" 32	6 00 "	1.5 "	"	Subcutaneous. Normal.	100 gm. meat.
	" 33	6 00 "	1.5 "	"	Subcutaneous. Normal.	150 gm. meat.
	" 34	6 00 "	1.5 "	"	meat during day.	"
	" 35	6 00 "	1.5 "	"	"	"

TABLE I—Continued.

Animal.	Date.	Time.	Blood calcium. mg. per 100 cc. serum	Extract given.	Remarks.
Dog 34. ♀ Cocker spaniel pup. 3.8 kilos.	1984				
	Oct. 24	4.00 p.m.		2 glands crude.	Thyroparathyroidectomy.
	" 25	1.00 "		2 "	Intravenous. Tetany onset.
	" 26	5.00 "		2 "	" Muscle tremors noted.
	" 27	9.00 a.m.			" Tetany.
		11.00 "			Normal.
	" 28	5.00 p.m.		2 "	Intravenous. Normal.
	" 29	4.30 "	6.8	2 "	" Tetany starting.
	" 30	6.00 "	6.9	2 "	" No tetany.
	" 31	6.00 "		2 "	By stomach tube. Normal.
				2 "	" " No tetany, but animal groaning.
	Nov. 1	5.00 "		2 "	By stomach tube. No tetany, but animal groggy.
Dog 35. ♂ Cocker spaniel pup. 3.9 kilos.	" 2	8.00 a.m.			Found dead.
	Oct. 24	4.30 p.m.		2 glands crude.	Thyroparathyroidectomy.
	" 25	5.00 "			Intravenous. Tetany.
	" 26	8.00 a.m.			Panting and spastic; no treatment.
		11.00 "			Found dead.
Dog 36. ♀ Brindle. 5.5 kilos.	Oct. 27	5.00 p.m.		4 glands = 82 per cent alcohol extract.	Thyroparathyroidectomy.
	" 28	5.00 "			Intravenous. No tetany.
	" 29	4.00 "	7.3	3.5 glands = 82 per cent alcohol extract.	Intravenous. Tremors.
	" 30	6.00 "		4 glands = 82 per cent alcohol extract.	By stomach tube. No tetany.

Oct. 31	6.30 p.m.		10 cc. = washings of isoelectric precipitate. 3 glands crude.	No tetany.
Nov. 1	6.30 "			By stomach tube. No tetany.
" 2				No extract.
" 3	1.00 "	5.5	5 cc. = solution of precipitate from 92 per cent alcohol.	Muscle tremors.
	4.30 "	6.9		Intravenous.
" 4	9.30 "	6.0	2 glands crude.	No tetany.
	9.30 a.m.	6.6		Intravenous. Strychnine-like convulsions immediately after injection. Artificial respiration.
	5.00 p.m.	6.2	4 " "	
				No tetany.
" 5	6.30 "	6.5		By stomach tube.
	10.30 "	8.8	3 " "	Subcutaneous. Tremors.
	10.00 a.m.	6.1	2 " "	By stomach tube. No definite tetany.
	3.30 p.m.	5.6	4 " "	
	10.30 "	6.4		By stomach tube. Twice daily.
" 6 to 13			2 to 3 glands crude.	No tetany.
" 14	11.00 a.m.		3 glands crude.	By stomach tube. Animal had been vomiting.
				Intravenous. Tetany.
" 15 to 19	4.00 p.m.	6.8	3 " "	By stomach tube.
	10.30 "		4 " "	Animal had refused bread and was considerably emaciated.
" 20 to 24			3 " " in 400 cc. milk daily.	No extract. Milk diet. No tetany.
" 25 to 28				500 gm. meat daily. No tetany.

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium. <i>mg. per 100 cc. serum</i>	Extract given.	Remarks.
Dog 37. ♀ Spaniel. 19 kilos.	1924 Oct. 29 " 30 " 31 Nov. 1 " 2 " 3 " 4 " 5 " 6 " 7 to 10 " 11	12 00 m.	10.8	1.5 glands isoelectric filtrate. 2 " " " 3 " " " No extract. 3 glands crude. 3 " " No extract. 3 " " No extract. 12.5	Thyroparathyroidectomy.
		4 00 p.m.	8.8		Intravenous.
		6 30 "			By stomach tube. Normal.
		6 30 "			" " "
		9 30 "	9.2		Normal.
		9 30 a.m.	10.1		Intravenous.
		8 30 "	10.2		Normal.
		4 00 p.m.	10.7		800 gm. meat. Normal.
		1 00 "			
		9 30 "			By stomach tube.
		10 00 a.m.			200 gm. meat daily. No tetany.
					Returned to kennel. No tetany to date, Nov. 28.
Dog 38. ♀ Collie. 10 kilos.	Oct. 29 " 30 " 31 Nov. 1 " 2	5 00 p.m.	11.5	2 glands isoelectric filtrate. 2 " " " 3 " " " 3 " 91 per cent alcohol extract. 1 30 p.m. 6 30 " of isoelectric precipitate. 8 00 a.m.	Thyroparathyroidectomy.
		6 00 "	8.3		Intravenous. Normal.
		3 30 "	7.4		" Tetany starting.
		6 30 "	8.7		
		8 30 a.m.			By stomach tube. Violent tetany.
		12 00 m.	6.2		Intravenous. Tetany all morning.
		1 30 p.m.			Some improvement.
		6 30 "	5.2		Never free from tetany all day.
		8 00 a.m.			Found dead.

Dog 39. ♀ Collie pup. 11 kilos.	Nov. 3	11.30 a.m.	12.0	<p>6 glands crude.</p> <p>5 cc. dialysate of unknown strength.</p> <p>3 glands isoelectric filtrate.</p> <p>3 " crude.</p> <p>3 " "</p> <p>3 " "</p> <p>10 cc. 10 per cent CaCl_2.</p> <p>3 glands crude.</p> <p>3 " "</p> <p>3 " "</p> <p>1.30 p.m.</p> <p>12.00 m.</p> <p>10.00 a.m.</p>	<p>Thyroparathyroidectomy.</p> <p>Intravenous. Tetany starting.</p> <p>" Tremors.</p> <p>Intravenous. By stomach tube.</p> <p>" " " Normal.</p> <p>" " " "</p> <p>Intravenous. Violent convulsions. By stomach tube. No tetany.</p> <p>" " " Violent convulsions. Artificial respiration.</p> <p>Intravenous. Some improvement.</p> <p>" No tetany since 4.00 p.m.</p> <p>Intravenous. Tetany. Died in strychnine-like convulsions immediately after alkaline extract injected.</p>
	" 4	9.30 p.m.	10.9		
		9.30 a.m.	8.8		
		7.00 p.m.	8.4		
	" 5	10.00 "	10.1		
		10.00 a.m.	8.7		
		11.30 "	8.9		
		2.20 p.m.	6.5		
		6.00 "			
	" 6	11.00 "	7.0		
		9.00 a.m.			
	" 7	9.30 p.m.	5.7		
Dog 40. ♀ Mongrel. 10 kilos.	" 8	9.30 a.m.	6.1	<p>6 glands crude.</p>	<p>Thyroparathyroidectomy.</p> <p>Intravenous.</p>
		6.00 p.m.			
	" 9	10.00 a.m.			
		1.30 p.m.			
		12.00 m.			
		10.00 a.m.			
		11.30 a.m.	12.9		
	Nov. 3	9.30 p.m.	9.9		
	" 4	9.30 a.m.	9.4		
		7.00 p.m.	6.9		
		10.00 "	10.7		

TABLE I—Continued.

Animal.	Date.	Time.	Blood calcium. <i>mg. per 100 cc. serum</i>	Extract given.	Remarks.
Dog 40—Cont.	194				
	Nov. 5	10.30 a.m.	8.4	3 cc. dialysate of unknown strength.	Intravenous.
		2.30 p.m.	8.6	2 glands isoelectric filtrate.	" Tremors.
	" 6	11.00 "	7.7	3 " crude.	By stomach tube.
		9.30 a.m.		3 " "	" " Normal.
	" 7	9.30 p.m.		3 " "	" " "
		12.30 a.m.	6.7	30 cc. dialysate of unknown strength.	Intravenous. Tremors.
		6.00 p.m.	7.0	5 glands crude.	By stomach tube.
	" 8 to 10			2 to 3 glands crude.	" " twice daily.
	" 11	10.00 a.m.		3 glands crude.	Normal.
		7.00 p.m.		2 " 82 per cent alcohol extract.	By stomach tube. Tremors.
	" 12 to 13			2 " 82 " "	" " " twice daily.
	" 14 to 16			2 " crude.	Normal.
	" 17	10.30 a.m.		2 " isoelectric filtrate.	" "
		6.00 p.m.		2 " "	Subcutaneous. 500 gm. meat.
	" 18	10.30 a.m.		2 " "	" 500 gm. meat.
	" 19	9.30 p.m.		2 " "	" 500 gm. meat.
		1.00 "		2 " "	" 250 gm. meat.
		3.30 "	16.7	3.30 "	Animal appears weak and depressed.
		6.30 "		6.30 "	Subcutaneous.
	" 20	10.00 a.m.	15.3	No extract.	Very dull and depressed. Refused food.
	" 21	8.00 "	14.0		Animal dead.

Dog 41. ♂ Mongrel. 12 kilos.	Nov. 7	3.00 p.m.			Thyroparathyroidectomy. By stomach tube. Normal.
	" 8	1.00 "		3 glands crude.	Intravenous. Tremors.
	" 9	11.30 "		3 "	Half intravenous. Half by stomach tube. Tetany.
		10.00 a.m.		" "	" "
	" 10	10.00 p.m.		6 "	By stomach tube. Tetany.
		10.00 a.m.		2 "	Intravenous. Condition not improved.
		11.30 "		3 "	" "
	" 11	7.00 p.m.		3 "	Intravenous. No tetany.
		10.00 a.m.	6.6	3 "	" Tremors.
		4.00 p.m.	8.6	2 "	No tetany.
		7.00 "		" 82 per cent alcohol extract.	By mouth. No tetany.
	" 12	10.00 a.m.		1.5 glands crude.	Intravenous. Tetany pronounced.
		10.15 "	6.7	3 "	" "
				" 80 per cent alcohol extract.	
		2.40 p.m.	8.8	2 glands 82 per cent alcohol extract.	Considerable improvement.
		7.00 "		4 glands 82 per cent alcohol extract.	By mouth.
	" 13	9.00 a.m.	5.4	4 glands 82 per cent alcohol extract.	" " Tetany. Considerably better after 3 hrs.
		5.00 p.m.	4.8	4 glands 82 per cent alcohol extract.	Intravenous. Tetany.
	" 14	6.00 "		7 cc. = water solution of precipitate from 82 per cent alcohol by 4.5 volumes of ether.	Able to walk.
		10.15 a.m.	5.7		

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium. <i>mg. per 100 cc. serum</i>	Extract given.	Remarks.
Dog 41—Cont.	1924 Nov. 14	2 30 p.m.	7.6		No tetany.
		6.15 "	7.3		Intravenous.
		10 20 "	7.3	7 glands crude.	
	" 15	8.30 a.m.		" "	By stomach tube. No tetany.
	" 16	6.00 p.m.		" "	" " Slightly spastic.
	" 17	10.00 a.m.		" "	" "
	" 17	10.00 p.m.	4.5	isoelectric filtrate.	Intravenous. Tetany.
	" 17	10.30 a.m.		" "	Subcutaneous. No tetany.
	" 18	6.00 p.m.		" "	" "
	" 18	10.30 a.m.		" "	" 500 gm. meat.
		6.00 p.m.	11.0		Normal.
	" 19	9.30 "		" "	Subcutaneous. Normal.
	" 19	1.00 "	9.8	" "	" 250 gm. meat.
	" 20	6.30 "		" "	" Normal.
	" 20	11.20 a.m.		" "	" Refused food.
	" 21	6.00 p.m.		1 gland	" Drowsy.
	" 21	11.00 a.m.	15.4		Drowsy. Refused food.
		3.20 p.m.	13.0	25 cc. 10 per cent NaHCO ₃ .	Intravenous.
		3.25 "	10.7		
	" 22	5.10 "	9.5		
	" 22	1.30 "	7.5	3 glands isoelectric filtrate.	Subcutaneous. Signs of tetany.
	" 23				Labored breathing. Muscles hy-
		5.00 "			perirritable.
		9.00 a.m.			Somewhat improved.
					Dead.

Dog 42. ♂ Terrier. 11 kilos.	Nov. 7	4.00 p.m.	3 glands crude.	Thyroparathyroidectomy. By stomach tube.
" 8	1.00 "	2	" "	" "
" 9	11.30 "	1	" "	" "
" 10	10.00 a.m.	1 gland	" "	" "
" 10	10.00 p.m.	2 glands	" "	" "
" 10	10.00 a.m.	1 gland	" "	" "
" 11	7.00 p.m.	2.5 glands	" "	Intravenous. Tetany starting.
" 11	10.00 a.m.	3 "	" "	" Tremors.
" 11	7.00 p.m.	6.7	" "	By stomach tube.
" 12	11.00 a.m.	2	82 per cent alcohol extract.	Intravenous.
" 12	11.00 a.m.	8.6	3 glands 80 per cent acid alcohol extract.	Tremors.
" 12	3.15 p.m.	7.8	2 glands 82 per cent alcohol extract.	By stomach tube.
" 12	7.00 "			" "
" 13	9.00 a.m.	5.7	3 glands 82 per cent alcohol extract.	Tremors.
" 13	5.30 p.m.	5.7	5 glands 82 per cent alcohol extract.	" "
" 13	7.00 "			" "
" 14	11.00 a.m.	5.7	5 cc. = precipitate from 82 per cent alcohol by 4.5 volumes of ether.	" "
" 14	2.30 p.m.	6.0	10 cc. = water extract of precipitate from 82 per cent alcohol.	" "
" 14	6.00 "	7.2	4 glands crude.	Intravenous. Spastic.
" 15	10.20 "	4.9	4 "	By stomach tube. No tetany.
" 15	9.00 a.m.		4 "	" " Spastic.
" 16	6.00 p.m.		4 "	" " Normal.
" 16	10.00 a.m.		4 "	" " "
" 16	10.30 p.m.	2	2 "	" " "

TABLE I—Continued.

Animal	Date.	Time.	Blood cal- cium.	Extract given.	Remarks.
Dog 42—Cont.	194				
	Nov. 17	10.30 a.m.		2 glands isoelectric filtrate.	Subcutaneous. Normal.
	" 18	1.00 p.m.		3 " "	" "
	" 18	10.30 a.m.		3 " "	500 gm. meat.
	" 19	9.30 p.m.		2 " "	Normal.
	" 19	1.00 "		3 " "	Tremors. Refused food.
	" 20	5.00 "	7.9	3 " "	Subcutaneous. No tetany. Re-
	" 20	6.30 "		3 " "	fused food.
	" 21	11.00 a.m.		3 " "	" "
	" 21	6.30 p.m.		1.5 " "	" "
	" 22	9.00 a.m.		2 " "	Ate 125 gm. raw meat.
	" 22	6.00 p.m.		2 " "	" 250 " " "
	" 23	6.00 "		2 " "	" 250 " " "
	" 23	8.00 "		2 " "	" 150 " " "
	" 24				No extract. 230 gm. meat.
	" 25	6.00 "		1 gland	Subcutaneous. Tremors. 230 gm. meat.
	" 26	4.25 "	11.2	4 glands	Subcutaneous. No food eaten.
	" 27	12.10 a.m.	13.7	1 gland	" " "
	" 28	7.00 p.m.		1 gland	Normal.

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium.	Extract given.	Remarks.
	1924		mg. per 100 cc. serum		
Dog 45. ♂ Mongrel pup. 3 kilos. Large goiter.	Nov. 7	6.00 p.m.		1 to 2 glands crude.	Thyroparathyroidectomy. By stomach tube. Twice daily. No tetany.
	" 8 to 16				Subcutaneous. Twice daily. " 100 gm. meat. " "
	" 17 to 18	1.00 "		1 to 1.5 " isoelectric filtrate.	
	" 19	6.30 "		1.5 glands isoelectric filtrate.	
	" 20			2 " "	
	" 21	8.30 a.m.	18.0	No extract. No food.	Very weak and muscles toneless all day. Dead. Blood from heart.
Dog 46. ♀ Mongrel pup. 3 kilos. Large goiter.	Nov. 8	1.00 p.m.		1 to 1.5 glands crude.	Thyroparathyroidectomy. By stomach tube. Twice daily. No tetany.
	" 9 to 12				Died suddenly after showing distress in breathing for 2 hrs. No real tetany until immediately prior to death. Bled from heart.
	" 13	6.00 "	6.0		
Dog 47. ♂ Mongrel pup. 3 kilos. Large goiter.	Nov. 8	1.30 p.m.		1 to 2 glands crude.	Thyroparathyroidectomy. By stomach tube. Twice daily. No tetany.
	" 9 to 14				By stomach tube. Subcutaneous. Tetany.
	" 15	9.00 a.m.		2 glands crude.	By stomach tube. No tetany.
		4.00 p.m.		2.5 " "	" " "
		6.00 "		4 " "	" " "
	" 16	10.00 a.m.		3 " "	" " "

	Nov. 17 to 18	10.30 p.m.		2 glands crude. 1 gland isoelectric filtrate.		By stomach tube. No tetany. Subcutaneous. Twice daily. No tetany. Subcutaneous. 100 gm. meat. " Normal. Animal comatose. Bled from heart. Died.
Dog 48. ♀ Collie. 17 kilos.	" 19	1.00 "		1.5 glands "		
	" 20	6.30 "		2 " "		
		9.00 a.m.	17.8			
		9.30 p.m.				
	Nov. 10	11.30 a.m.		3 glands crude.		Thyroparathyroidectomy.
	" 11	7.00 p.m.	8.8	3 " "		Intravenous. Panting. Spastic.
		10.00 a.m.	9.0			"
		4.00 p.m.	9.5	4 " 82 per cent alcohol ex-tract.		By stomach tube.
	" 12	10.00 "	8.1	3 glands acid alcohol extract.		Intravenous. Tetany.
		12.30 "	7.9	4 " crude extract.		" No improvement.
		6.00 "	7.2	3 " "		" Early in afternoon decided improvement in condition.
	" 13	9.00 a.m.	7.3	4 " 82 per cent alcohol ex-tract.		6 p.m. Worse again.
		5.30 p.m.	6.7	5 glands 82 per cent alcohol ex-tract.		By stomach tube. No tetany.
	" 14	7.00 "		3 " crude extract.		" " Tremors.
		10.30 a.m.	7.1	6 " "		Intravenous. Violent tetany.
	" 15	2.45 p.m.	9.5			" No tetany.
		10.30 "	8.2	5 " "		" " "
		9.00 a.m.		6 " "		By stomach tube. No tetany.
		6.00 p.m.		8 " "		" " "

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium. <i>mg. per 100 cc. serum</i>	Extract given.	Remarks.
Dog 48—Cont.	1934 Nov. 16 " 17 " 18 " 19 " 20 " 21 " 22 " 23 " 24 to 28	10.00 a.m.	6.3	5 glands crude extract.	Intravenous. Violent tetany.
		3.00 p.m.	7.0		
		10.00 "			
		10.00 a.m.		5 " isoelectric filtrate.	Subcutaneous.
		6.00 p.m.		2.5 " "	"
		10.30 a.m.		5 " "	"
		9.30 p.m.		3 " "	"
		12.40 a.m.		3 " "	"
		4.45 p.m.	13.2		250 gm. meat.
		6.30 "		2.5 " "	"
		11.00 a.m.		2.5 " "	"
		10.40 "	11.3		200 gm. meat.
		6.00 p.m.		2.5 " "	Refused food.
		6.00 "		2.5 " "	" "
		3.40 "	7.8	2.5 " "	Tremors. Refused food.
		9.20 "	10.7		Refused food.
Dog 49. ♀ Mongrel. 12 kilos.	Nov. 17 " 18 " 19	11.30 a.m.	10.6		Subcutaneous. Daily. Eating very little. No tetany.
		3.45 p.m.	7.9	Extract of 1/3 gm. desiccated gland.	Thyroparathyroidectomy.
		9.00 "	8.3		Intravenous. Tremors.
		11.30 "	5.9	3.5 glands isoelectric filtrate.	No tremors.
		11.30 a.m.	8.3	3 " "	Subcutaneous. Tremors.
					" 250 gm. meat.
					Normal.

	Nov. 20	5.30 p.m. 6.30 " 10.20 a.m.	8.3	2.5 glands isoelectric filtrate. " " " "	Subcutaneous. " 250 gm. meat. Normal. " "
	" 21	5.30 p.m. 10.00 a.m. 6.00 p.m.	7.0 6.8	" " " " " "	250 gm. meat. Subcutaneous. 250 gm. meat. Tetany. " "
	" 22	6.00 "	6.8	" "	Subcutaneous. 250 gm. meat.
	" 23	4.20 "	8.2	" "	Normal.
	" 24	10.00 "	10.3	" "	Normal.
	" 25	6.30 " 6.00 "	2 2	" " " "	" 500 gm. meat during day. " 500 " "
	" 26	3.00 "	7.3	" "	" Tetany. 500 gm. meat during day.
	" 27	10.30 " 1.20 " 7.00 "	8.1 7.6	" "	Subcutaneous. No tetany. 500 gm. meat during day.
	" 28	a.m.	2	" "	Normal.
Dog 50. 9 Spaniel. 12 kilos.	Nov. 17	12.15 p.m.	10.7	5 glands acid alcohol extract. " isoelectric filtrate. " " " "	Thyroparathyroidectomy.
	" 18	4.20 " 8.40 "	7.4 8.2		Tremors.
	" 19	11.25 " 12.00 m.	6.3 9.75		No tremors.
					Subcutaneous. Tremors.
					" 250 gm. meat. Normal.

TABLE I—Continued.

Animal.	Date.	Time.	Blood calcium. <i>mg. per 100 cc. serum</i>	Extract given.		Remarks.
Dog 50—Cont.	1924					
	Nov. 19	4.15 p.m.	10.2	2.5 glands isoelectric filtrate.		Subcutaneous. Normal.
	" 20	6.30 "	8.9	" "		" 250 gm. meat.
		11.15 a.m.				Normal.
	" 21	6.00 p.m.	10.0	2.5 " "		" "
		10.00 a.m.		No extract.		250 gm. meat. Normal.
		5.30 p.m.	6.6	3.5 glands isoelectric filtrate.		Subcutaneous. 250 gm. meat.
	" 22					Tetany.
		a.m.				Normal.
		6.00 p.m.	5.3	2.5 " "		Subcutaneous. 250 gm. meat.
	" 23					Tetany.
		a.m.				Normal.
		8.30 p.m.	5.9	2.5 " "		Subcutaneous. 250 gm. meat.
						Tetany.
		10.00 "	5.9			Tremors.
	" 24	10.00 a.m.	9.8			Normal. 250 gm. meat.
		6.30 p.m.	5.7	2 " "		No definite tetany. 500 gm. meat during day.
	" 25	6.00 "		2 " "		Subcutaneous. Normal. 500 gm. meat during day.
	" 26	3.30 "	6.4	3 " "		Subcutaneous. Tetany. 500 gm. meat during day.
		10.45 "	8.1			Complete recovery.

	Nov. 27	12.20 a.m. 1.15 p.m. 4.30 "	8.9 6.6 6.1	3 glands isoelectric filtrate.	500 gm. meat during day. Subcutaneous. Tetany. Normal.
Dog 51. ♀ Mongrel. 16 kilos.	Nov. 17	12.45 p.m.	11.2	6 glands 82 per cent alcohol extract precipitated with ether.	Thyroparathyroidectomy. Tremors.
	" 18	4.00 "	8.0		
		8.45 "	9.6		
	" 19	11.40 "	8.1	3 glands isoelectric filtrate.	Subcutaneous.
		11.40 a.m.	10.2	3 " "	" 250 gm. meat.
		4.10 p.m.	11.2		
		6.30 "		3 " "	"
	" 20	10.45 a.m.	10.1	2.5 " "	" 250 gm. meat.
		5.45 p.m.	9.0	2.5 " "	" 250 "
	" 21	10.00 a.m.		No extract.	250 gm. meat.
		6.00 p.m.	8.0	2.5 glands isoelectric filtrate.	Subcutaneous. 250 gm. meat.
	" 22	6.00 "	8.2	2.5 " "	" 250 "
	" 23	3.30 "	9.2	2 " "	" 250 "
	" 24	9.30 "	14.4	2.5 " "	" 250 "
		6.00 "	15.4		Died. Drowsy all day. Would not eat.
Dog 52. ♀ Mongrel. 16 kilos.	Nov. 17	1.15 p.m.		7 glands precipitate from 82 per cent alcohol with 9 volumes acetone.	Thyroparathyroidectomy.
	" 18	4.40 "	7.40		
		8.45 "	7.9	2 glands isoelectric precipitate.	Subcutaneous.
		11.15 "	7.2		

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium.	Extract given.	Remarks.
Dog 52—Cont.	1944		<i>mg. per 100 cc. serum</i>		
	Nov. 19	12.20 p.m.	7.8	3 glands isoelectric precipitate.	Subcutaneous. 250 gm. meat.
		4.15 "	9.3		
	" 20	6.30 "		" "	"
		11.10 a.m.	8.6	" "	"
	" 21	6.00 p.m.	9.0	" "	"
		10.00 a.m.			250 gm. meat.
	" 22	5.30 p.m.	8.0	" "	250 "
	" 23	6.00 "	6.8	" "	250 "
		4.00 "	8.8	" "	250 "
	" 24	9.40 "	13.9		250 "
		9.00 a.m.			250 "
	" 25	6.30 p.m.	2	" "	250 "
	" 26	5.00 "	2	" "	Subcutaneous. Normal. 500 gm. meat during day.
	" 27	4.00 "	3	" "	"
	" 28	7.8			
		10.00 "	13.5		
		12.00 m.	14.2		
	" 27	10.45 a.m.	12.1		
		6.00 p.m.			
	" 28		2	" "	"
					Normal.

TABLE I—Continued.

Animal.	Date.	Time.	Blood cal- cium.	Extract given.	Remarks.
Dog 54. ♂ Collie. 18 kilos.	1924		<i>mg. per 100 cc. serum</i>		
	Nov. 21	2.30 p.m.			Thyroparathyroidectomy.
	" 22				No tetany.
	" 23	9.30 a.m.	5.7	10 cc. = ether-alcohol extract 10 glands.	Intravenous. Tremors.
		3.30 p.m.	5.7		Mild tetany.
	" 24	8.30 "	5.7	10 cc. = solution of precipitate from ether-alcohol extract used above.	Tetany marked.
		10.00 a.m.	6.2		Tremors.
		6.00 p.m.	5.3	3 glands isoelectric filtrate.	Subcutaneous. Tetany.
	" 25	8.30 a.m.	9.5	3 " "	" 500 gm. meat during day.
		10.00 "	8.0		
		1.00 p.m.	8.0		
		4.00 "	9.0		
		6.00 "	7.0	1.5 " "	Subcutaneous.
	" 26	10.45 a.m.	7.3	9 " "	" Tetany.
		11.15 "	7.3		
		11.45 "	7.9		
		1.10 p.m.	8.5		
		2.50 "	9.4	" "	"
		4.45 "	10.1	" "	"
		6.35 "	8.6	" "	"
		9.45 "	9.1		500 gm. meat during day.
		12.00 m.	10.6		

	Nov. 27	9.30 a.m. 1.30 p.m. 6.00 "	9.6 11.4 9.9	2 glands isoelectric filtrate.	Subcutaneous. 500 gm. meat during day. Normal.
	" 28				
Dog 55. ♂ Collie. 20 kilos.	Nov. 21	3.30 p.m.			Thyroparathyroidectomy.
	" 22				No tetany.
	" 23	9.30 a.m.	7.9	10 cc. = 90 per cent alcohol extract 15 glands.	Intravenous. 250 gm. meat. Normal.
		3.30 p.m.	6.8		No tetany.
		9.00 "	7.7		" "
	" 24	10.00 a.m.	7.7	2.5 glands isoelectric filtrate.	" " 250 gm. meat during day.
		6.00 p.m.	6.1		Subcutaneous. No tetany. 250 gm. meat.
	" 25	6.00 "		1.5 " " "	Subcutaneous. 500 gm. meat during day.
	" 26	12.00 a.m. 3.45 p.m. 10.15 "	8.1 7.5 9.1	4 " " "	Subcutaneous.
		11.55 " 10.45 a.m. 6.00 p.m.	14.7 10.1	2 " " "	500 gm. meat during day.
	" 27				Subcutaneous. 500 gm. meat during day.
	" 28				Normal.
Dog 56. ♂ Normal collie. 19.5 kilos.	Nov. 26	10.30 a.m. 11.00 " 11.40 " 1.05 p.m.	10.9 11.7 11.7 13.5	9 glands isoelectric filtrate.	Subcutaneous.

TABLE I—*Concluded.*

Animal.	Date.	Time.	Blood cal- cium.	Extract given.	Remarks.
	1944		<i>mg. per 100 cc. serum</i>		
Dog 56— <i>Cont.</i>	Nov. 26	2.40 p.m.	13.7	4 glands isoelectric filtrate.	Subcutaneous.
		3.00 "		3 "	"
		6.30 "	12.7		
		9.30 "	14.2		
	Nov. 27	9.30 "	15.2		
		1.20 "	12.2		
		6.00 "	12.7		
Dog 57.	Nov. 27	10.30 a.m.	11.1	10 glands isoelectric filtrate.	Subcutaneous.
♂ Normal col- lie. 18 kilos.		1.00 p.m.	12.6		
		3.25 "	13.7		
		6.00 "	14.5		
Dog 58.	Nov. 27	10.30 a.m.	11.1	5 glands isoelectric filtrate.	Subcutaneous.
♀ Normal col- lie. 15.5 kilos.		1.00 p.m.	11.1		
		3.30 "	12.1		
		6.00 "	11.7		
Dog 59.	Nov. 27	10.30 a.m.	10.7	2.5 glands isoelectric filtrate.	Subcutaneous.
♂ Normal mongrel. 21.5 kilos.		1.00 p.m.	10.4		
		6.30 "	9.9		
Dog 60.	Nov. 27	10.30 a.m.	11.8	1.5 glands isoelectric filtrate.	Subcutaneous.
♂ Normal col- lie. 11 kilos.		1.10 p.m.	13.2		
		3.40 "	13.2		
		6.30 "	13.0		

TABLE II.

Dog No.	Amount of extract given.		Method of administration.	Blood calcium.		Time between analyses.
				Before.	After.	
	cc.	glands		mg. per 100 cc. serum	mg. per 100 cc. serum	hrs.
31	5	2	By stomach.	6.9	9.2	12
32	5	2	Intravenous.	7.5	10.0	5
	2		"	5.5	6.6	5½
33	4	2	"	5.6	10.8	3
	4	2	"	5.9	6.4	26
	5	3	"	4.1	5.2	7½
36	5		"	5.5	6.9	3½
	3	2	"	6.0	6.6	12
	8	5	"	6.2	8.8	5½
	4	2	Subcutaneous.	5.6	6.4	7
38	10		Intravenous.	7.3	8.6	3
39	5	3	"	6.0	7.0	5
40	6	6	"	6.9	10.7	3
			Subcutaneous.		16.7	
41	10	3	Intravenous.	6.7	8.6	6
		3	"	6.7	8.7	4½
		4	"	4.7	5.7	19
	7		"	5.7	7.6	4
			Subcutaneous.	4.5	11.0	Cumulative 48.
			"	9.7	15.4	" 48.
42	8	4	"	11.2	13.7	8½
48	12	6	Intravenous.	7.1	9.5	4
	10	5	"	6.3	7.0	5
	5	2.5	Subcutaneous.	7.8	10.7	5
49	7	3.5	"	5.9	8.3	12
	4	2	"	8.2	10.3	6
	6	3	"	7.3	8.1	7½
50	6	3	"	6.3	9.8	12½
	5	2.5	"	8.9	10.0	7
	4	2	"	5.9	9.8	12
	6	3	"	6.4	8.1	8
					8.9	9½
51	6	3	"	8.1	10.2	12
	6	3	"	10.2	11.2	4½
	5	2.5	"	9.2	14.4	6
52	4	2	"	7.2	7.8	13
	6	3	"	7.8	9.3	4
	5	2.5	"	8.6	9.0	7
	4	2	"	6.8	8.8	21
	4	2	"	8.8	13.9	6½

TABLE II—*Concluded.*

Dog No.	Amount of extract given.		Method of administration.	Blood calcium.		Time between analyses.
				Before.	After.	
	cc.	glands		mg. per 100 cc. serum	mg. per 100 cc. serum	hrs.
52	6	3	Subcutaneous.	7.8	13.5	6
					14.2	8
					12.1	19
53	40		Intravenous.	7.8	8.3	3
	6	3	Subcutaneous.	7.0	10.9	4
	4	2	"		13.4	5½
54	6	3	"	5.9	9.6	
	6	3	"	5.3	9.5	14
	18	9	"	7.3	9.4	4
55	5	2.5	"	6.1	8.1	18
	8	4	"	7.5	9.1	6½
					14.7	8
56	20	10	"	10.9	10.1	19
					13.7	4
					13.7	4
57	10	5	"	11.1	13.7	4
58	5	2.5	"	11.1	12.1	4
60	2.5	1.25	"	11.8	13.2	4

1. Replacement Therapy.

It is generally agreed that complete parathyroidectomy in dogs is fatal unless dietary treatment, calcium therapy, or intravenous saline injections are instituted at once. Factors which favor the early onset of fatal tetany are lack of preoperative care of animals and the feeding of meat after operation. The administration of alkaline salts such as sodium bicarbonate may cause tetanic convulsions in normal animals. Such salts are more prone to produce this effect in parathyroidectomized animals.

As stated previously the animals received no special dietary treatment prior to operation. In the later experiments a heavy meat diet has been started at once after recovery from the operation. The extracts used in a large number of experiments were definitely alkaline. In fact on two occasions tetanic convulsions have been observed immediately following the intravenous injection of certain potent extracts. This was due no doubt to the action of alkali in the extract *per se*.

Conditions favorable to the development of tetany have been

existent in these experiments. The prevention or control of tetany in such experiments would therefore point to the presence in the extracts used of a chemical entity or hormone which is capable of exercising when introduced into the blood the same functions that the parathyroid gland exercises normally. Dogs 32 and 33 furnish an excellent example of complete replacement therapy. These animals are now, about 5 weeks after operation, seemingly perfectly normal. They have been "proven up" from time to time as the protocols illustrate. They have had different potent extracts administered and these have been shown to be effective either by the oral route or by injection, intravenous or subcutaneous. Moreover, they have recently been placed on a heavy meat diet. In the earlier experiments meat was definitely avoided and animals received only bread and water at that time. The later work shows quite definitely, however, that a heavy meat diet is not in the least to be feared, provided potent extract is administered in adequate amount. Dogs 49, 50, 51, 52, 53, 54, and 55 illustrate this very well.

2. Effect upon Blood Calcium.

Parathyroid tetany and a low level of blood calcium have long been recognized as coincident phenomena. That the low calcium in itself is the cause of tetany has not been universally granted. However, the work of Salvesen would seem to point to this being the case. Also, the fact that calcium chloride injections will absolutely control or prevent tetany in parathyroidectomized animals would seem to place the cause of tetany in the low calcium content of the blood serum.

Coincident with the marked clinical improvement following the injection of potent parathyroid extract into animals in a condition of tetany and having a low blood calcium, a rise in the level of blood calcium has been observed time and time again. It is possible to have an animal with a low blood calcium manifest no obvious clinical signs of tetany. The effectiveness of a parathyroid extract can be ascertained by determining the blood calcium curve following its administration to an animal with a low blood calcium even though the usual manifestations of tetany be absent. It is possible that an assay of a parathyroid extract will ultimately be made on the normal animal by following the calcium curve,

after administration of the extract, in much the same manner as insulin is assayed. The experiments with normal dogs, Nos. 56 to 60, illustrate that the calcium content of the blood serum can be definitely elevated by potent parathyroid extract. A surprising result was obtained in Dogs 57 to 60. It will be noted that, whereas graded doses were used on the animals in this series, a relatively small dose was almost as effective in raising the calcium as a large one. It is probable therefore that the calcium content of the blood may be most effectively raised by small doses of the extract repeated at intervals of a few hours. A number of experiments in which the effects of the extract on normal animals are being determined are now in progress and will be reported later.

The calcium content of the extracts which have been used in this investigation is negligible. The isoelectric filtrate, which was very potent, was of such strength that 2 cc. were the equivalent of one ox parathyroid gland, and contained 0.09 mg. of calcium per cc. of extract. This extract also contained 1 mg. of non-protein nitrogen and 1 mg. of protein nitrogen per cc. The glands used were found to be quite uniform in size and averaged 0.25 gm. in weight.

In Table II are shown the effects of the administration of potent parathyroid extracts upon blood serum calcium.

It is of interest to note that the rise in blood serum calcium content does not take place immediately following the administration of the extract. There is a definite latent period, and the most marked effects are only manifested after some hours.

It would appear that the close parallelism of blood serum calcium values and of clinical conditions is no mere coincidence since it has been consistently present throughout this series of experiments.

A point of great interest in this work was the experimental production of a new entity—hypercalcemia. Overdosage with the parathyroid hormone has resulted in death with high blood calcium. Whether the high calcium is the immediate cause of death in such cases remains to be determined. The clinical manifestations of hypercalcemia in dogs appear to be loss of appetite, dullness, drowsiness verging on coma, general atonia, and a failing circulation. The animals are also obviously dehydrated, the blood becomes very concentrated, and it becomes a matter of

great difficulty to secure serum from blood samples for analysis even when a high power centrifuge is used. Immediately after death the blood in the heart and great vessels forms a firm clot. Hypercalcemia was the probable cause of death in Dogs 34, 40, 41, 43, 47, and 51.

The case of Dog 41 is of particular interest in that tetany was frequently present and very low levels of blood calcium were observed. The tetany was absolutely controlled when adequate doses of the hormone were administered. A meat diet had no ill effect and hypercalcemia was induced by overdosage. Sodium bicarbonate injection was also shown to be effective in lowering blood calcium in this animal. An analysis of this animal's blood after death was not made as the serum could not be obtained even after long centrifugalization.

The experiments taken as a whole show that tetany can be avoided in parathyroidectomized dogs if a potent extract is administered once or twice daily after the operation. The later work in which more potent extracts were used shows that the dosage must be sufficient, but no more than sufficient, if hypercalcemia is to be avoided. The experiments show also that if tetany develops in a parathyroidectomized animal, it can be completely relieved by parathyroid extract. Moreover, they illustrate that parathyroidectomized animals which have been kept free from tetany by the use of parathyroid extract can be thrown into tetany even several weeks after the operation by removing or decreasing the extract. The factor of diet would seem, in the light of these experiments, to be relatively insignificant in the pathogenesis of parathyroid tetany. The normal function of the parathyroid glands would appear to be related to the regulation of certain phases of mineral metabolism through a direct control of the calcium level of the blood. While it may later develop that these glands have other functions, there would seem to be no need at present to ascribe any function to them other than that of regulators of calcium metabolism. Loss of this function following removal of the glands is the sole cause of tetania parathyreopriva in the opinion of the writer.

It has been quite conclusively shown that the extract may be effective by the oral route. It is felt, however, that best results are to be obtained by subcutaneous injection; that is, that the full

effect of small doses is better elicited by this mode of administration of the hormone. The sterilized isoelectric filtrate which has been used in this way has caused no local reaction in the experimental animals. The extract is crystal-clear. The active principle is apparently insoluble in ether, but soluble in alcohol up to at least 92 per cent concentration. The time, temperature, and concentration of acid which will give the best yield of potent material have as yet to be ascertained. After a method of producing a potent extract had been evolved it was thought advisable to adhere to it more or less rigidly during the early stages of the investigation. There still remains much to be done in the way of standardizing the preparation and further purifying it. The glands have been worked up as a rule in lots of 75 or 100. On a few occasions, glands which have been preserved in anhydrous acetone have yielded potent extracts when treated according to the technique described above. Desiccated material has not given satisfactory results in our hands.

It is possible that this hormone will have many practical uses. Calcium metabolism enters into many medical problems. An agent is now at hand which influences profoundly the calcium metabolism. It must be recognized, however, that the parathyroid hormone is a most potent therapeutic agent and that its use may be attended with great danger unless due precautions are taken to avoid an overdose and the development of hypercalcemia. It may be that a condition of mild hypercalcemia would be of definite benefit in certain conditions. Only by careful clinical studies, however, will the ultimate merits or demerits of this hormone be determined.

SUMMARY.

1. An extract has been made from the parathyroid glands of oxen by the use of which parathyroid tetany in dogs can be prevented or controlled.
2. The active principle in this extract produces its effect by causing the calcium content of the blood serum to be restored within normal limits.
3. A very close parallelism has been observed between the clinical condition of experimental animals and the calcium content of the blood serum. Coincident with the marked improvement

observed following the use of the active extract a rise in blood calcium has been noted.

4. Overdosage effects have been observed and the blood findings in this condition invariably show a condition of hypercalcemia.

5. The symptoms of hypercalcemia are anorexia, vomiting, apathy, drowsiness verging into coma, and a failing circulation.

6. Hypercalcemia in parathyroidectomized dogs is a fatal condition if allowed to persist.

7. Sodium bicarbonate has been observed to reduce the calcium content of the blood serum in hypercalcemia.

8. Tetania parathyreopriva has been prevented or controlled in dogs receiving no preoperative preparation and which have been placed on a heavy meat diet immediately following recovery from the operative procedure. Animals in which tetany has been prevented from occurring by prophylactic treatment have been thrown into tetany by temporary withdrawal of the treatment.

9. One to two treatments per day are sufficient to prevent tetany in parathyroidectomized dogs.

10. The extract containing the active principle has been found to be effective by each of three modes of administration; namely, by the oral route, by intravenous injection, and by subcutaneous injection.

11. A rise in the level of blood calcium in the normal dog has been observed following the injection of parathyroid extract.⁴

It is a pleasure to acknowledge the invaluable assistance of Dr. E. P. Clark who carried out the calcium determinations herein reported, also of Dr. J. W. Scott who assisted in the surgical part of the work.

I have also to acknowledge the financial assistance kindly afforded by a grant from the Carnegie Foundation to which my best thanks are due.

⁴ Since the submission of the data herein recorded for publication, the papers of Dr. Hanson of Fairbault, Minnesota, published in the *Military Surgeon* (Hanson, A. M., *Mil. Surg.*, liv, 76, 218, 554), have come to the attention of the writer. Hanson's attempts to prepare an active extract of the parathyroid glands of animals are worthy of great commendation. His clinical results are indeed highly suggestive.

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THE EFFECT OF A PARATHYROID HORMONE ON NORMAL ANIMALS.

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INTRODUCTION.

In a previous communication (1) it was shown that tetany in parathyroidectomized dogs could be prevented or controlled by the administration of a special extract of the parathyroid glands of the ox. It was also shown that this extract exercised a direct control of the concentration of calcium in the blood and that coincident with the marked improvement in the clinical condition of parathyroidectomized dogs following the administration of the parathyroid hormone there was observed an elevation of the level of blood calcium. It was noted that the calcium content of the blood rose gradually, but very definitely, over a period of some hours, that a maximum point was finally reached, and that a return to lower levels of calcium concentration was gradually accomplished. In each experiment of this nature there was therefore a definite blood serum calcium curve somewhat indicative of the effectiveness of the preparation used in treatment.

It seemed to us that the final proof of the existence in potent form of the active principle of the parathyroid gland, in the extracts used in the early experiments, had been obtained by the successful replacement therapy controlled by blood calcium studies earlier reported. The next problem which was studied, therefore, was the effect of these potent extracts upon the normal animal. The results of a number of experiments in which our extract has been administered to normal animals are reported in this communication.

Methods.

Normal dogs were used. The extract was administered by subcutaneous injection. Blood samples for analysis were taken at frequent intervals from a leg vein. Calcium was estimated in the blood serum by the Kramer-Tisdall method (2) with slight modifications. Phosphorus was estimated on a few samples by the Briggs method (3). Alkali reserve was determined on occasion by the Van Slyke method (4), using whole blood equilibrated with alveolar air. The extracts containing the hormone were made as previously reported.

RESULTS.

The effect of single injections of the extract into normal dogs upon the blood serum calcium values is shown graphically in Chart 1. Blood serum calcium curves following a single injection of the extract into parathyroidectomized dogs are shown for comparison in Chart 2.

The effect of two injections of the extract into normal dogs is shown in Chart 3, while in Chart 4 the curves obtained for blood serum calcium following two injections of the extract into parathyroidectomized dogs are given.

The effect of successive injections of graded doses of the extract into six normal dogs is shown in Chart 5.

In Charts 6 to 12 are shown the effects of repeated doses of the extract at different intervals.

The results of the administration of the extract to normal animals by stomach tube are shown in Chart 13.

DISCUSSION.

Effect of a Single Injection of the Extract into a Normal Dog.

The effect of a single injection of the extract into a normal dog is, in as far as the blood serum calcium curve may be used as an index, much the same as the effect of a single injection into a parathyroidectomized animal. There is a gradual rise in blood serum calcium values which reaches a maximum in from 5 to 9 hours and then follows a return to normal, the slope of the curve being almost the same as that of the earlier rising curve. By comparison, the effect in these experiments on blood serum cal-

cium value of a single injection of the extract into parathyroidectomized dogs is more marked. The initial rise in calcium content of the serum was usually greater than was the case in normal animals, and the return to the original level was more prolonged. This observation was in keeping with our experience in treating parathyroidectomized dogs with the extract. In the

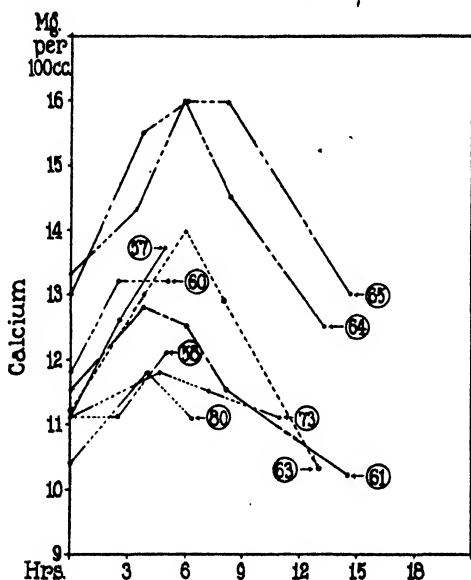


CHART 1.

Dog 57, ♂	18	kilos,	20	cc. extract	= 10	glands.
" 58, ♀	15	"	10	"	= 5	"
" 60, ♂	11	"	2.5	"	= 1.25	"
" 61, ♀	16.5	"	3	"	= 1.5	"
" 63, ♂	16	"	5	"	= 2.5	"
" 64, ♂	14	"	10	"	= 5	"
" 65, ♂	22	"	10	"	= 5	"
" 73, ♀	7.5	"	2.5	"	= 1	gland.
" 80, ♂	12.5	"	2.5	"	= 1	"

majority of cases it has been found that a single daily injection is sufficient to ward off tetany. A few animals, as the protocols published in a previous communication will indicate, require treatment at less than 24 hours intervals. Two injections daily into such animals are not without danger, however, as over-dosage phenomena may be encountered.

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Effect of Two Injections of the Extract into Normal and Parathyroidectomized Dogs.

The effect of two injections of the extract is largely dependent upon the time interval between the injections. In the normal dog, if the second injection is administered early on the rising calcium curve, the general character of the curve is very little altered.

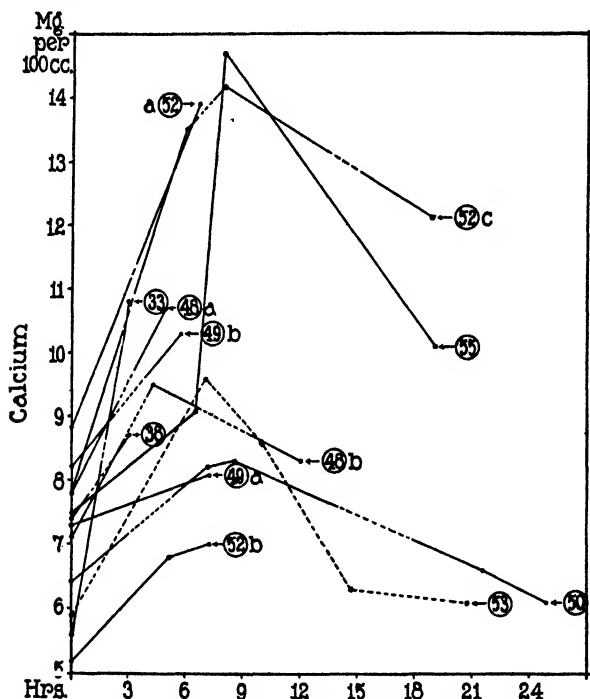


CHART 2. Dog 33, ♂ 4 kilos,	4	cc. extract = 2	glands.
38, ♀ 10 "	10	2	"
48, ♀ 15 "	(a) 5	2.5	"
	(b) 12	6	"
49, ♀ 12 "	(a) 6	3	"
	(b) 4	2	"
50, ♀ 12 "	6	= 3	"
52, ♀ 16 "	(a) 4	= 2	"
	(b) 2.5	= 1	gland.
	(c) 6	= 3	glands.
53, ♀ 12 "	6	= 3	"
55, ♂ 20 "	8	" "	= 4

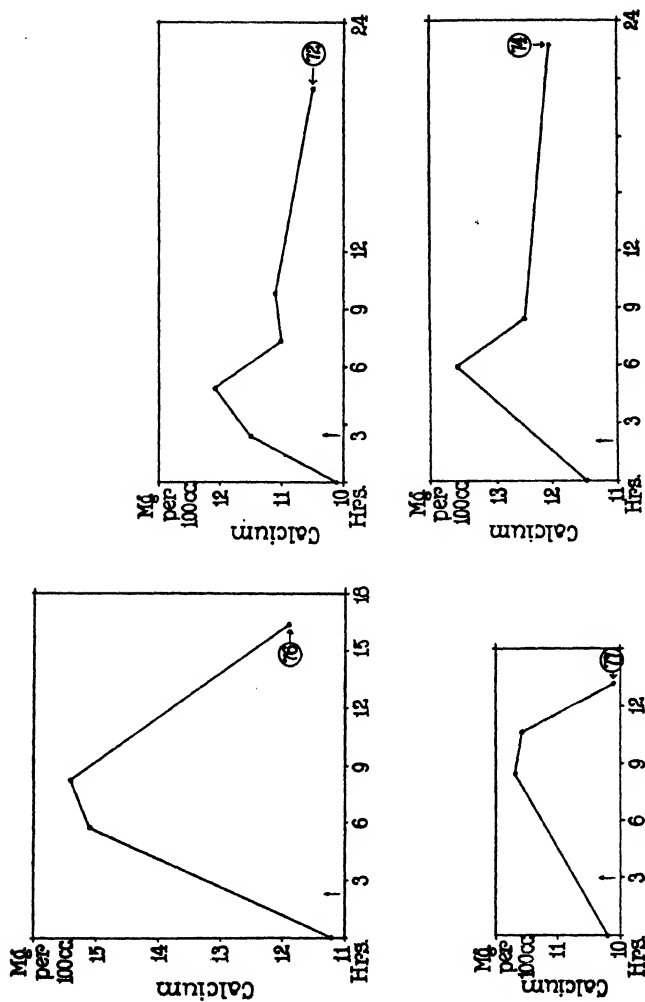


CHART 3. Dog 72, ♀ 8 kilos, 2 cc. extract = 1 gland. Potency test.

" 74, ♂ 10 " 2.5 " special extract = 4 glands.

" 76, ♀ 12.5 " 2.5 " extract = 1 gland. Passed through Mandler filter.

" 77, ♀ 18.5 " 1st injection 4 cc.; 2nd injection, 2.5 cc. Potency test.

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Slightly higher values for blood serum calcium may result, and the return to normal is somewhat more prolonged. If the second injection is made at or near the peak of the curve following the single injection, a dip in the curve will most likely result before the second injection has begun to affect materially the level of blood serum calcium. Pyramiding or cumulative action may be most strikingly demonstrated by such treatment (Chart 4). Dog

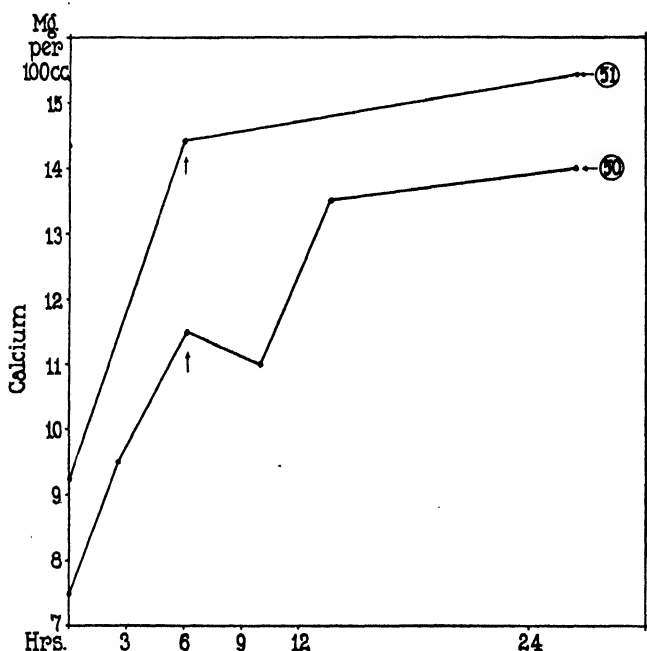


CHART 4. Dog 50, ♀ 12 kilos, 2 cc. extract = 2 glands. 2 injections
 " 51, ♀ 16 " 4 " " = 2 " 2 "

51 showed typical symptoms of hypercalcemia during the day following the two injections and finally died at 6 p.m. of that day, Dog 50, on the other hand, made a complete recovery, and is still alive. This animal has frequently been allowed to develop tetany and has in every instance completely recovered from such attacks following a single injection of the extract.

Effect of Successive Injections of the Extract into Normal Dogs.

When several injections of the extract are made into normal dogs at intervals of a few hours the phenomenon of pyramiding is manifested in the blood serum calcium curve. The blood serum calcium under such circumstances rises to a very high level. If injections be discontinued the blood serum calcium values may gradually approach normal or death may intervene when hypercalcemia is still manifested. Even after injections have been discontinued and the blood serum calcium content has fallen to lower levels, death may still occur in the course of 24 to 48 hours, probably as the direct result of the condition of extreme hypercalcemia earlier existent. The results of six experiments carried out simultaneously on six normal dogs are shown in Chart 5. Each animal was injected at approximately 2 hour intervals with the same extract. The dosage used varied from 1 to 6 cc. of a preparation, 2 cc. of which were equivalent to one ox parathyroid gland. When symptoms became very pronounced injections were discontinued. Two dogs in this series died, the other four made a complete recovery. It would appear that blood serum calcium values above 15 mg. per 100 cc. are essential before definite symptoms of hypercalcemia (hormone over-dosage) are manifested. The animals gradually become weak. There is as a rule diarrhea and vomiting, and, in fatal cases, blood-stained fluid may be vomited or passed by the bowel. The condition of atonia in fatal cases becomes more marked, and complete collapse ultimately follows. The circulation becomes impaired, and in some instances it is almost impossible to get blood samples even when venesection is resorted to. It becomes increasingly difficult to secure serum from blood specimens, and blood taken at death has on occasion been centrifuged for an hour without any appreciable separation of the cells from serum taking place. Accurate viscosity measurements have not been made in these experiments, but by rough methods it is evident that there is at least 100 per cent increase in blood viscosity in the terminal state. There is, however, no great change in hemoglobin values. 5 to 15 per cent increases in hemoglobin have been noted, which, in view of the frequent bleedings, would indicate a definite decrease in plasma volume, but yet nothing like sufficient decrease to account for the profound change noted in

the character of the blood. The blood chemistry in hypercalcemia due to parathyroid hormone overdosage promises to be a most interesting study. In this communication we are only able to report a few results on this subject. The problem is being studied in a systematic manner, however, and a detailed report will be made of the findings at a later date.

*Relationship Between Size of Dose of the Extract and the
Physiological Effect.*

The physiological effect of the extract administrations would seem to be more dependent upon the time interval between injections than upon the size of dose, provided always that a known potent preparation of the extract is used and something more than the minimal effective dose is administered. The blood serum calcium curves obtained on six animals above referred to and shown graphically in Chart 5 illustrate this phenomenon exceptionally well. The dosage used in this series of experiments varied 600 per cent, and it is interesting to note that the two cases which ended fatally in this instance received 2 and 6 cc. doses, respectively, while complete recovery occurred in the four animals which received 1, 3, 4, and 5 cc. doses, respectively. The general character of the blood serum calcium curves is the same in all instances. Chart 11, which illustrates the blood serum calcium curve obtained in our first experiment of this nature, shows that the effect of a single massive dose (18 cc. = 9 ox glands) is very little different from the effect of a much smaller dose (see Chart 1). The decrease in blood calcium obtained in this animal some 3 hours following the second injection is of interest. It is suggestive of a latent period and in this instance this would correspond with the passing off of the effect of the first dose. A third injection resulted in pyramiding and a great prolongation of the period of hypercalcemia. No ill effects were noted in this animal.

The extract has been administered to a number of human subjects, and blood serum calcium values have been increased thereby. The minimal effective dose in the human individual is probably about one-third to one-half an ox gland. Definite rises in blood calcium have been obtained with 1 cc. doses (= 0.5 gland), while no effect has been obtained with single 0.5 cc. doses of the same preparation on the same subject.

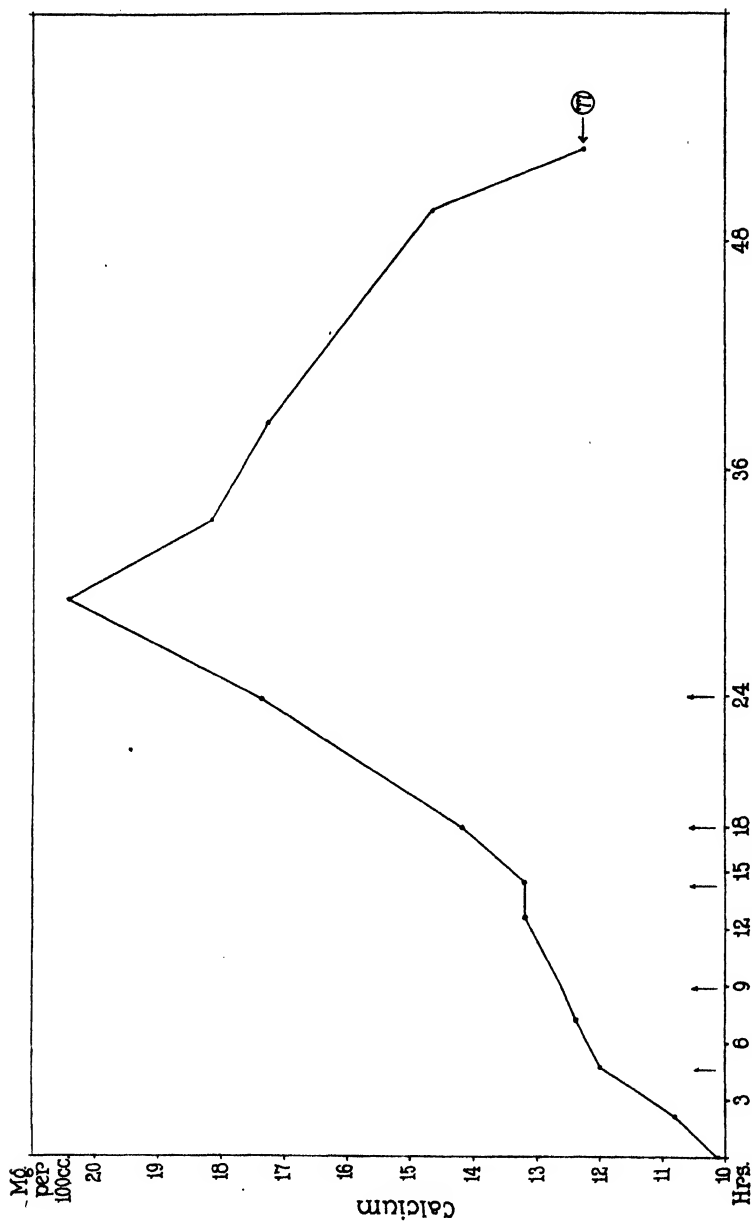


CHART 6. Dog 77, ♀ 18.5 kilos, 6 cc. extract = 2 glands injected at intervals indicated by arrows on the chart.

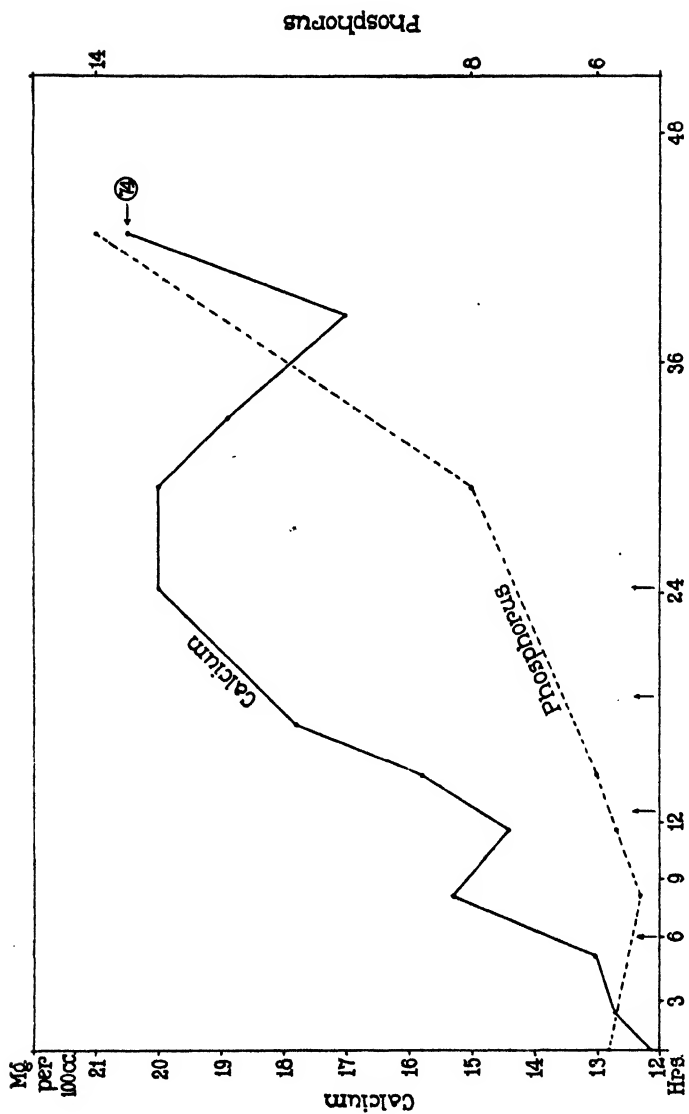


CHART 7. Dog 74, ♂ 10 kilos, 6 cc. extract = 2 glands injected as indicated by arrows on the chart. The broken line indicates phosphorus, the solid line calcium values.

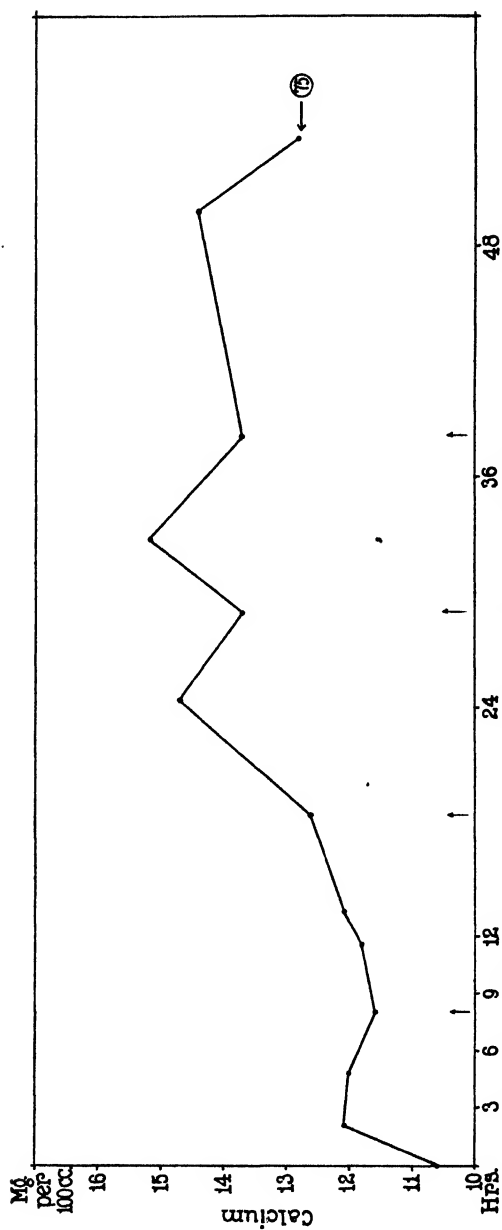


CHART 8. Dog 75, ♀ 10.5 kilos, 6 cc. extract = 2 glands injected as indicated by arrows on the chart.

*Effect of Varying the Interval between Successive Injections
of the Extract into Normal Animals.*

The effect of varying the interval between successive injections of the extract into normal animals is shown in Charts 6 to 12, inclusive. Dog 77 (Chart 6) received 6 cc. of extract (= 2 ox glands) in each of six injections spaced over 24 hours. The blood calcium rose steadily to 20.5 mg. per 100 cc. during this period, injections were then discontinued, and the animal made a complete recovery. Dog 74 (Chart 7) received the same dosage as Dog 77 administered five times over 24 hours. This animal died. There was a premortal fall in blood serum calcium in this instance followed by a subsequent rise. Phosphorus was determined on blood serum samples in this animal and the sharp rise in the phosphorus curve coincident with the preterminal fall in calcium is suggestive of a protective response on the part of the organism to combat the extreme hypercalcemia. Phosphates have been shown to lower blood calcium (5), and a mobilization of phosphorus may well be a natural protective response in hypercalcemia.

Dog 75 (Chart 8) received five injections, each equivalent to two ox glands, during 38 hours. No ill effects were noted, and pyramiding is not so pronounced as in the case of Dogs 77 and 74. A condition of mild hypercalcemia was, however, maintained over a period of many hours. Should this hormone have a clinical use it is just this sort of result, mild but continued hypercalcemia, which should be aimed at in the treatment.

The experiment on Dog 78 (Chart 9) is very instructive. This animal was injected with 6 cc. of extract (= 2 glands) on three occasions during 18 hours. It will be noted that the effect of the first injection had almost passed off when the second injection was made at 11½ hours. The third injection was made, however, while the effect of the second was at or about its maximum. The result was marked pyramiding and a greatly prolonged period of hypercalcemia. Dog 79 (Chart 10) represents a somewhat similar experiment. The result here is suggestive of cumulative action and is indicative of the fact that pyramiding may be obtained in normal animals even with two injections per day. 2 days later when this animal had an almost normal blood serum calcium value (11.6 mg.), 2 cc. injections (= 0.66 of an ox

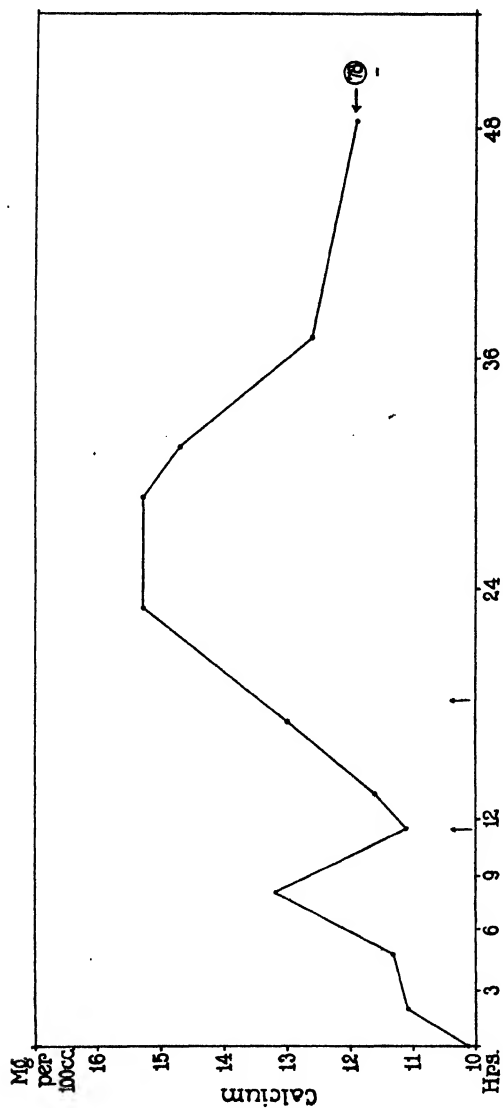


CHART 9. Dog 78, ♀ 18 kilos, 6 cc. extract = 2 glands injected as indicated by arrows on the chart.

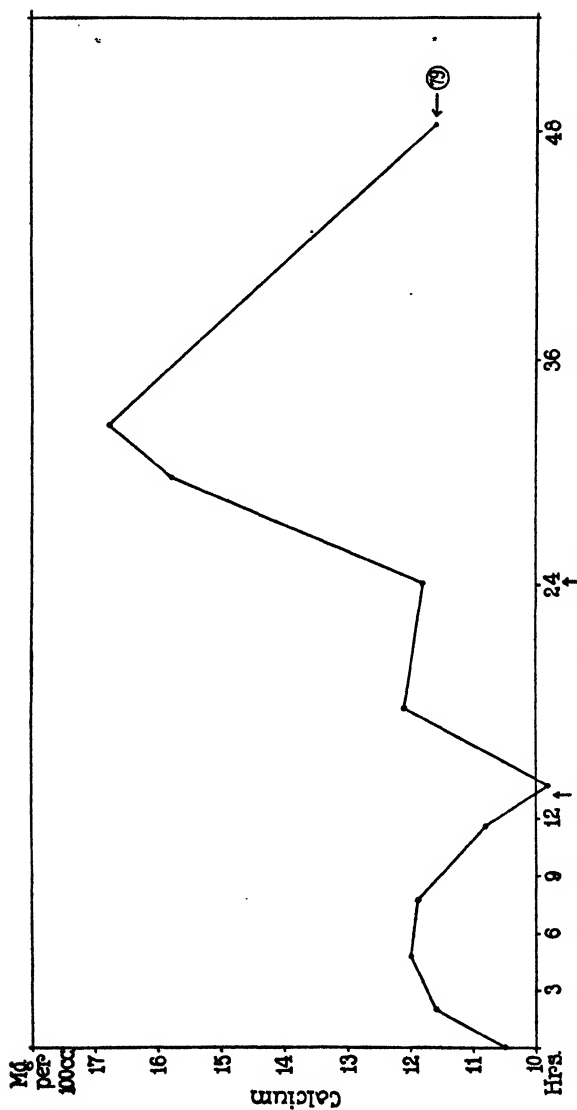


CHART 10. Dog 79, ♂ 31.5 kilos, 6 cc. extract = 2 glands injected as indicated by arrows on the chart.

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gland) were made every 20 minutes for 13 hours. At the end of 8 hours the animal had diarrhea, was vomiting, but was otherwise normal. The blood calcium curve it will be noted is very little different from a number of others (Chart 5). There is evidently a limit to the rate at which calcium can be mobilized even though injections be made as frequently as was the case in this experiment. At the end of 13 hours injections were discontinued. The animal showed signs of general weakness during the next 2 days, but it was anticipated that a recovery might be made. Death, however, supervened. It was absolutely impossible to secure serum from the blood specimen taken shortly after death, so an analysis for calcium could not be made. Phosphorus was determined on whole blood, and the result was 13 mg. per 100 cc. The values for phosphorus shown on the chart represent analyses on serum, the final value of 13, being on whole blood, has been omitted from the graph. This same blood sample had a urea nitrogen of 111 mg., a non-protein nitrogen of 120 mg., creatinine 3.5 mg., and creatine 6 mg. per 100 cc., and a chlorine calculated as sodium chloride of 0.330 per cent.

Blood Chemistry at Death Following Hypercalcemia.

As yet we have been able to make only a few studies of the variations in various blood constituents which are induced by parathyroid hormone overdosage resulting in death. This part of the problem promises to be full of matters of great interest and it is now being studied in a systematic manner. In addition to the calcium curve in parathyroid hormone overdosage it is now proposed to determine the curve for halogen, sodium, potassium, magnesium, urea, non-protein nitrogen, total nitrogen, and osmotic pressure, as determined by the cryoscopic method.

The following data relating to the blood chemistry in fatal hypercalcemia have already been obtained.

1. *Halogens.*—Five cases only have been studied. In each instance there has been observed a gradual fall in defibrinated blood halogen which was most pronounced in the terminal state.

2. *Urea Nitrogen.*—The urea nitrogen has risen in each experiment and has reached a very high level at death.

3. *Non-Protein Nitrogen.*—This has paralleled somewhat urea nitrogen.

4. *Cryoscopic Measurements.*—In four cases studied there has been a

depression in the Δ of 0.09-0.14°C. at the terminal state as compared with controls.

5. *Blood Sugar*.—This has been found to be within the normal range.

6. *Viscosity*.—An enormous increase in viscosity has always been observed.

7. *Protein Content*.—No exact measurements of protein content have been made, but it is a significant fact that the usual Folin-Wu tungstic acid precipitation technique applied to defibrinated blood, taken terminally has given at times highly colored filtrates which could be avoided by the use of double amounts of 10 per cent sodium tungstate and 2/3 N sulfuric acid.

8. *Alkali Reserve*.—This has not been followed to a great extent as yet, but it would appear that there is little or no change in this value early in the condition but that a marked drop occurs terminally.

The following results have been obtained in four cases.

Analysis.	Control.	Terminal.
Dog 90.		
	mg.	mg.
Halogen.....	315	286
Urea nitrogen.....	16	75
Non-protein nitrogen.	43	162
Δ	0.61°C.	0.75°C.
Dog 86.		
Halogen.....	320	270
Urea nitrogen.....	14	63
Non-protein nitrogen.	41	166
Δ	0.60°C.	0.71°C.
Dog 87.		
Halogen.....	315	257
Urea nitrogen.....	14	72
Non-protein nitrogen.	41	219
Δ	0.63°C.	0.74°C.
Dog 89.		
Halogen.....	335	273
Urea nitrogen.....	10	72
Non-protein nitrogen.	26	184
Δ	0.59°C.	0.72°C.

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The enormous increases in non-protein nitrogen and urea nitrogen occur only many hours after hypercalcemia has been established and are to be regarded as preterminal phenomena. The decrease in halogen is a matter of great interest because it may be by slight changes in the chlorine content of the blood that a diuretic action and a decrease in edema has been manifested in the human individual treated with the extract.

Blood Serum Phosphorus during Parathyroid Hormone Overdosage.

As yet only a few determinations of blood serum constituents other than calcium have been determined in experimental hyper-

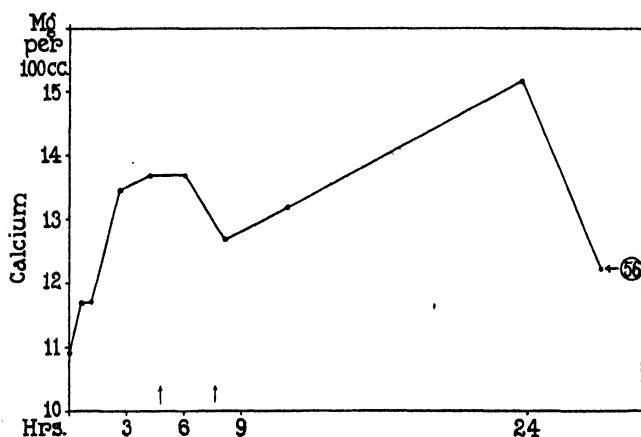


CHART 11. Dog 56, ♂ 20 kilos, 1st injection 18 cc. = 9 glands; 2nd injection 8 cc. = 4 glands; 3rd injection 6 cc. = 3 glands.

calcemia. The results which have so far been obtained on phosphorus values for blood serum during parathyroid hormone overdosage would indicate that there is very little change during the early hours of hypercalcemia. If the condition of high blood serum calcium is maintained, however, and a fatal issue finally results, the blood serum phosphorus rises markedly. The cases of Dogs 74 (Chart 7) and 79 (Chart 12) have already been referred to. The blood serum of Dogs 62 and 66 (Chart 5) was

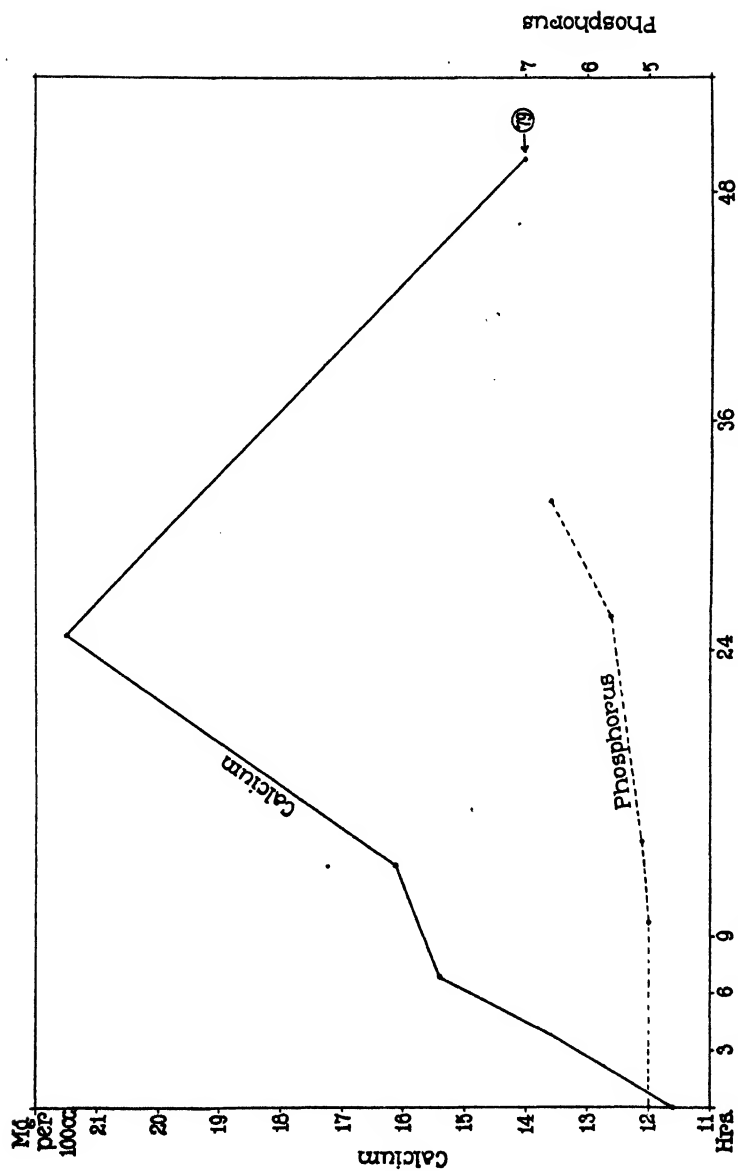


CHART 12. Dog 79, ♂ 31.5 kilos, 2 cc. = 0.66 gland injected every 20 minutes for 13 hours.

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analyzed for phosphorus and in each case a terminal rise was noted. In the case of Dog 62

At 10 hours, blood serum calcium was 15.9 mg. and phosphorus 5.6 mg.
 " 17 " " " " " 16.4 " " 5.7 "
 " 20 " " " " " 17.6 " " 5.7 "
 " death, calcium was 17.2 mg. and phosphorus 8.3 mg.

In the case of Dog 66

The control calcium was 10.3 mg. and phosphorus 4.9 mg.
 At 4 hours, " " 13.4 " " 5.5 "
 " 10 " " 14.6 " " 6.5 "
 " death, " " 16.5 " " 9.9 "

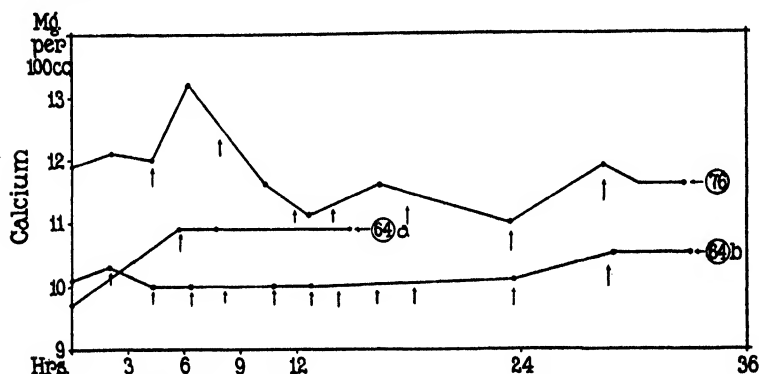


CHART 13. Dog 64, ♂ 18 kilos, a 5 cc. extract = 2 glands by stomach tube as indicated by arrows on the chart. (b) 6 cc. extract = 2 glands by stomach tube as indicated by arrows on the chart. Dog 76, ♀ 12.5 kilos, 6 cc. extract = 2 glands by stomach tube as indicated by arrows on the chart.

These experiments indicate that the phosphorus content of the blood serum tends to rise during hypercalcemia, but more especially in the later stages of this condition.

Effect of the Extract Administered to Normal Dogs by Stomach.

In view of the fact that definitely positive results had previously been obtained both in parathyroidectomized dogs and in man by the oral administration of the extract, it was somewhat surprising to find that it is practically impossible to produce overdosage effects in normal dogs by the intermittent administration of the extract by stomach tube. Chart 13 is illustrative of three experiments of this type. The explanation of such results was readily obtained, however, when known potent preparations

were submitted to the action of pepsin and trypsin. In all instances where this has been done, there has been almost complete loss of potency of the preparations so treated. The effectiveness of oral administration, therefore, must lie in rapid absorption and so circumvention of enzyme action. Subcutaneous administration of the extract is in our opinion the most effective and also the most readily controllable.

Standardization of the Extract.

Physiological standardization of the extract would seem to be possible. It is too early as yet to attempt to define a unit. The influence of the weight of the animal on the blood serum calcium curve has yet to be definitely determined. The minimal effective dose on a dog of definite weight would seem to be something to strive to determine. In our experience known potent preparations (that is tested on parathyroidectomized dogs in tetany) are capable of causing an elevation in the level of blood calcium in the normal dog.

A few dogs, which might in a sense be termed refractory, have been encountered in which little appreciable change in blood serum calcium content has been manifested following a single injection. Such animals have invariably shown hypercalcemia when the injections of the extract have been continued. Successive administrations of small doses at intervals of 2 to 4 hours are, therefore, recommended in potency testing. Several blood serum calcium determinations should then be made, and error thus reduced to a minimum.

It is felt that all parathyroid hormone preparations should be tested physiologically before using. Up to the present, the dosage has been expressed in terms of the average ox gland. In other words the fresh glands have been counted out and extracted. The gram basis will be used in the future, however.

SUMMARY.

1. The effect of the subcutaneous and oral administrations of a parathyroid hormone to normal dogs is reported.
2. It has been found that a potent extract, administered by subcutaneous injection, causes an elevation in the level of blood

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serum calcium. The change so produced in calcium values follows a typical curve. These curves have been contrasted with similar curves obtained on parathyroidectomized dogs.

3. The effect of successive injections of a potent extract into normal dogs has been studied. A condition of profound hypercalcemia has thereby been produced. Such a condition when long maintained may result in death.

4. The effect of varying the dosage and varying the time of successive administrations has been studied.

5. The blood in fatal cases of hypercalcemia shows very definite changes of both a physical and chemical nature.

It is a pleasure to acknowledge the financial assistance afforded to the carrying out of this work by a grant from the Carnegie Foundation.

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A STUDY OF THE TISDALL METHOD FOR THE DETERMINATION OF BLOOD SERUM CALCIUM WITH A SUGGESTED MODIFICATION.

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In connection with the work being done on the parathyroid hormone in this laboratory, it became necessary to adopt a method for the determination of calcium in blood that would be as simple as possible and at the same time give consistent results. The method of Kramer and Tisdall,¹ modified later by Tisdall,² meets the latter requirement provided the directions are closely followed. On the other hand, after some preliminary experiments, it was found that any variation in procedure gave results which differed by several per cent, and it was not possible to recover entirely added calcium. Thus it was apparent that the method was arbitrary—a conclusion one would also arrive at from purely chemical reasoning when the solubility of calcium oxalate in pure solvent and in the presence of oxalate ions is considered. A study was therefore made of the method, with the object of shortening the procedure, if possible, and of finding the conditions that would constantly give the actual amount of calcium present in blood serum. The criterion adopted for the demonstration of the latter point was that, within the limit of experimental error, complete recovery of calcium was obtained from a calcium solution of known concentration, and, under the same conditions, known amounts of calcium added to blood serum were completely recovered. In order to realize these requirements, some 200 determinations were made under various conditions, including the use of different concentrations of ammonium oxalate as the precipitant, variation in the length of time

¹ Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, xlvii, 475.

² Tisdall, F. F., *J. Biol. Chem.*, 1923, lvi, 439.

allowed for precipitation with different concentrations of oxalate, different ways of washing the calcium oxalate precipitate, the use of a constant temperature bath in which to carry out the titrations, and of various amounts of serum for the determination. In no case were there less than four simultaneous determinations made for any one condition. Without reporting the large amount of experimental data obtained, it may be stated at once that when the various factors influencing the results are carefully considered, the conclusion arrived at is, that for the small amount of calcium present in blood serum the procedure which must be adopted, in order to give true values, is entirely arbitrary and must be closely adhered to.

As a result of the above mentioned study, Tisdall's method has been modified somewhat so as to meet the foregoing requirements. It is the purpose of this communication to record these details as the method adopted, and which is being used, in connection with the work on the parathyroid hormone reported elsewhere and now in progress in this laboratory.

The modified Tisdall method which has been selected is as follows:

2 cc. of clear serum, 2 cc. of distilled water, and 1 cc. of 4 per cent ammonium oxalate solution are thoroughly mixed in a 15 cc. graduated centrifuge tube of the type described by Tisdall. The mixture is allowed to stand for 30 minutes or longer³ and then centrifuged until the precipitate is well packed in the bottom of the tube. The supernatant liquid is carefully poured off, and, while the tube is still inverted, it is placed in a rack for 5 minutes to drain,⁴ the mouth of the tube resting on a pad of filter paper. The mouth of the tube is wiped dry with a soft cloth and the precipitate is stirred up and the sides of the tube washed with 3 cc. of dilute ammonia water (2 cc. of concentrated ammonia and 98 cc. of water), directed in a very fine stream from a wash bottle. The suspension is centrifuged and drained again as just described; then 2 cc. of approximately normal sulfuric acid are added. The acid is blown from

³ It was found, as reported by Tisdall, that with 4 per cent ammonium oxalate the precipitation is complete within 30 minutes. With a more dilute solution, however, longer time is required for complete precipitation.

⁴ To insure uniform drainage, the tubes are always cleaned thoroughly by heating at approximately 100° for a few minutes in a cleaning mixture made by adding 1,500 cc. of concentrated sulfuric acid to a solution of 200 gm. of sodium dichromate in 100 cc. of water.

the pipette directly upon the precipitate so as to break up the mat and facilitate solution. The tube and contents are placed in a boiling water bath for about 1 minute and the oxalic acid is then titrated with 0.01 N potassium permanganate, using a micro burette.⁶ In our experience the titration is best carried out in a water bath at a temperature of 70-75°. The bath used consists of an 800 cc. Pyrex beaker, filled with water and heated with a small coil of No. 30 gauge chromel wire, having a resistance of about 6.5 ohms. After the temperature reaches 70°, it is controlled by either a sliding or lamp bank rheostat.

The only difference between the above procedure and that given by Tisdall is that one washing with 3 cc. of ammonia water is substituted for two washings with 4 cc. each of dilute ammonia,

TABLE I.

No. of cc. of 0.9925 \times 0.01N potassium permanganate required for		
2 cc. CaCl ₂ solution containing 10 mg. of Ca per 100 cc.	2 cc. ox serum.	2 cc. ox serum — 0.1 mg. of calcium.
1.01	1.11	1.61
1.01	1.10	1.61
1.01	1.12	1.62
1.00	1.10	1.61
Average.....1.007 or 9.99 mg. Ca per 100 cc.	Average.....1.107 or 11.0 mg. Ca per 100 cc.	Average.....1.61 or 16.0 mg. Ca per 100 cc.

The serum therefore contains 11.0 mg. of calcium per 100 cc. From the calcium chloride solution 99.9 per cent of the calcium was recovered; and from the serum to which calcium was added, 100 per cent recovery was realized.

and the tubes are allowed to drain for 5 minutes. It was found that by draining the tubes as indicated, an amount of mother liquor is left in the tube of the order of 0.02 cc. as compared with about 0.1 cc. without draining. Thus, with one washing there remains ammonium oxalate equivalent to about 0.2 per cent of the calcium present in 2 cc. of normal serum, but the loss of calcium oxalate due to its solubility is much reduced, especially since the first wash water contains an appreciable amount of oxalate

⁶ We prefer to make the 0.01 N potassium permanganate, sufficient for the day's use, by diluting 0.1 N solution and checking this against a standard 0.01 N sodium oxalate solution.

⁶ McBride, R. S., *J. Am. Chem. Soc.*, 1912, xxxiv, 393. Blum, W., *J. Am. Chem. Soc.*, 1912 xxxiv, 1387.

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ions which tend to suppress the solubility of the calcium oxalate. The total result is that under these conditions the two errors balance each other. The results given in Table I were obtained by the above procedure, using an accurately prepared calcium chloride solution (made from calcite), ox serum, and the serum to which a known amount of calcium had been added.

In order to compare the above modification with Tisdall's method the same serum and reagents were used following Tisdall's directions. The results noted below were thus obtained.

2 cc. of serum required

1.07 cc. $0.9925 \times 0.01 \text{ N KMnO}_4$

1.07

1.05

1.07

Average 1.065 cc. or 10.58 mg. Ca per 100 cc.

It is our conviction from the results we have obtained in this study and from over a thousand determinations which have been made during the past $2\frac{1}{2}$ months in connection with the work on the parathyroid hormone that, when the procedure, as recorded above, is carefully carried out, the actual amount of calcium in blood serum may be obtained with an error of not more than 2 per cent.

CLINICAL CALORIMETRY.

XXXVII. INFECTION AND THE KETOGENIC BALANCE.

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(Received for publication, January 8, 1925.)

It is a well known fact that infection in diabetes is attended with a change of the patient for the worse. Among the manifestations of this change is an increase in the production of the acetone bodies. Thus, many, perhaps a majority, of the cases of diabetic coma occur with infection (Joslin, 1). The possible causes for this increase in acidosis are as follows: (a) there might be a change in the mechanism by which the acetone bodies are produced; and (b) without any such change there might be a decrease in the proportion of the glucose oxidized. Two of the diabetics observed by Wilder and Winter (2) had mild afebrile infections, and excreted more acetone bodies than could be accounted for by the ketogenic balance.

By means of an extensive literature the principles underlying the excretion of the acetone bodies have been fairly firmly established. Shaffer (3, 4) has written an able review of the subject, including his own contributions, and a short discussion will be found in an earlier paper from this laboratory (5). Ketosis results when there is in the metabolism an undue preponderance of substances which tend to produce ketosis over those which tend to prevent it. It has been shown that in non-infected individuals, diabetic or otherwise, the presence of ketosis depends mainly upon an excessive proportion of fat to carbohydrate in the metabolism. It is necessary to take into account also the oxidation of protein, which contributes both ketogenic and antiketogenic factors, that is to say, both keto acids and glucose. This may be done either by using the total respiratory quotient as measured

TABLE I.
Calorimeter Data in Terms of Averages per Hour.

Subject.* Age. Weight. Surface area.	Time of observation.	CO ₂ gm.	O ₂ gm.	R.Q.	N ₂ in urine. gm.	Indirect calories.	Average pulse.	Work† adder (aver- age).	Remarks.*
John H. Dec. 14, 1923. 77.4 kg. 1.92 sq. m.	11.45 a.m. to 2.45 p.m.	22.8 • 22.8	• 22.8	0.727	0.371	74.6	82	28	Diabetes. Bronchopneu- monia. <i>Bacillus mucosus</i> <i>capsulatus</i> septicoemia. Quiet.
Ellen S. Feb. 4, 1924. 48.6 kg. 1.48 sq. m.	12.45 p.m. to 3.45 p.m.	19.8	19.3	0.748	0.602	62.8	97	29	Diabetic gangrene. Glass of milk at 6 a.m. Quiet.
John B. Feb. 1, 1924. 53.3 kg. 1.63 sq. m.	12.43 p.m. to 3.43 p.m.	27.6	26.7	0.751	0.487	87.7	97	29	Infected skin. Asleep most of 1st hr.; restless during 3rd hr.
Alex De V. Feb. 18, 1924. 57.7 kg. 1.66 sq. m.	12.12 p.m. to 3.12 a.m.	27.6	26.9	0.746	0.430	88.2	103	27	Empyema. Restless during 2nd hr.
Feb. 19, 1924. 57.5 kg. 1.64 sq. m.	11.59 a.m. to 2.59 p.m.	27.0	26.5	0.740	0.529	86.7	102	42	Moderately restless.
Apr. 15, 1924. 58.4 kg. 1.67 sq. m.	11.31 a.m. to 2.31 p.m.	23.1	21.9	0.767	0.450	72.0		30	After recovery. Restless.

* All of the patients were in the postabsorptive state during the observations, except as otherwise indicated.

† The work adder is an instrument by which the activity of the patient is measured. A very quiet patient raises the work adder less than 5 cm., and a very restless patient more than 25 cm.

(Shaffer, 4) or by determining the quantities of protein, fat, and carbohydrate oxidized, using the data of a calorimeter observation, including the nitrogen of the urine. Applying the formula of Woodyatt (6), the fatty acid-glucose ratio of the materials undergoing oxidation may be calculated. Work in several laboratories, including this one (5) shows that ketosis can be avoided so long as the fatty acid-glucose ratio calculated from the foodstuffs metabolized is less than 1.5, or the respiratory quotient 0.76 or over. It should be pointed out that we are not making any attempt to account for the actual quantities of ketones excreted, but are concerned simply with the ketogenic balance at the threshold of ketosis.

Method.

Four patients were observed, all of whom had infections. Two of them had diabetes, and two did not. Of the diabetics, one had bronchopneumonia and septicemia, and the other infected gangrene of the leg. Of the non-diabetics one had an infection of the skin following pneumonia, and the other empyema. Except with the diabetics, it was necessary in order to produce ketosis to withhold carbohydrate from the diet for 3 or 4 days. A basal observation was then made with the calorimeter to determine the respiratory exchange. This, together with the nitrogen of the urine, gave data for calculating the quantities of protein, fat, and carbohydrate oxidized, from which the ketogenic balance could be deduced, using the formula of Woodyatt. The more important data are given in Table I.

It should be noted that although this formula was originally applied to the diet, we have used for the calculations only the respiratory exchange and the nitrogen of the urine. Comparison was then made between the urinary excretion of the acetone bodies and the ketogenic balance derived from the respiratory data, either directly by means of the observed respiratory quotient or indirectly by means of the formula of Woodyatt. The urine of the calorimeter period was analyzed, since it had been found in earlier work (5) that the results were essentially the same as when the total 24 hour specimen was used. This proved to be true of the data in this paper as well. In only one instance was a difference as great as 0.02 gm. per hour found, as the result of analyzing

the 24 hour specimen instead of the urine of the calorimeter period. The chemical methods used in the work were the same as those referred to in an earlier paper (7). Since in the paper on uncomplicated diabetes ketosis was arbitrarily defined as the excretion in 24 hours of 1 gm. or more of acetone bodies expressed as acetone, the same arbitrary threshold was used in this work. This corresponds to the excretion of 0.04 gm. per hour.

In two instances the carbon dioxide-combining power of the plasma was determined before and after the observation, in order to exclude changes in the fixed acid of the blood such as might drive off carbon dioxide and thereby increase the respiratory quotient. With John H. on December 17 it increased only from 56.7 to 57.6, and with Ellen S. on February 5 from 66.3 to 67.2. Such a change is too small to affect the respiratory quotient.

The accuracy of the calorimeter was tested by means of alcohol checks, the results of which will appear in tabular form in a subsequent paper (8).

Case Histories.

Case 1.—John H. Bronchopneumonia. *Bacillus mucosus capsulatus* septicemia. Severe diabetes. Salesman, born in the United States in July, 1860. Height 176.0 cm. He was admitted to the hospital on Dec. 12, 1923, and to the metabolism ward on the following day. Symptoms of diabetes had begun in Aug. of the same year. Physical examination showed him to be stuporous, with definite cyanosis. Diminished resonance and moist râles were found at the bases of both lungs posteriorly. The heart was somewhat enlarged. The knee jerks were absent. The temperature ranged about 101° during his stay in the metabolism ward. On Dec. 14 it varied from 100.0° to 100.8°, on Dec. 17 from 100.0° to 100.4° and on Dec. 18 from 100.2° to 100.6°. His condition improved somewhat, but on Dec. 25 he had a chill and very high fever, and died 3 days later. *Bacillus mucosus capsulatus* was demonstrated in the circulating blood on Dec. 27. The highest blood sugar was 375 mg. per 100 cc. on Dec. 14 and the lowest carbon dioxide-combining power of the blood plasma was 57 vol. per cent on Dec. 17.

Case 2.—Ellen S. Severe diabetes, with gangrene of the right foot. Housewife, born in Jan., 1871. Height 160.3 cm. She was admitted to the hospital on Feb. 2, 1924, and to the metabolism ward 2 days later. 2 years previous one of the left toes became sore, but healed spontaneously. She entered the hospital because of gangrene. The physical examination was essentially negative except for gangrene of the right foot extending to the ankle, and redness of the calf of the leg. The gangrenous area showed evidence of infection, and there was a septic fever, ranging from 104°

to 97°. On Feb. 4 the temperature varied from 101.0° to 101.2°, on Feb. 5 from 101.6° to 102.4°, and on Feb. 6 from 100.0° to 100.2°. On Feb. 6 she was transferred to the surgical wards, but the amputation which was performed failed to check the spread of the gangrene, and she died on Feb. 17. The maximum blood sugar was 466 on Feb. 4. At no time was sugar found in the urine while under observation in the metabolism ward. The greatest excretion of acetone bodies was 0.78 gm. in 24 hours.

Case 3.—John B. Lobar pneumonia, complicated by impetigo simplex. Taxi chauffeur, age 32 years, born in Ireland. Length 172.6 cm. He was admitted to the hospital on Jan. 8, 1924, and to the metabolism ward on Jan. 28. In the interval he ran the course of a typical lobar pneumonia. On Jan. 16 note was made of a pustular eruption on the back, diagnosed as impetigo simplex. On Jan. 29 the temperature was rising, and there were confluent pustules over a wide area of skin on the back and buttocks. The patient complained of pain in the wrists, which on the next day became tender and painful, as did several finger joints. The temperature, which had reached a maximum of 103.6° on Jan. 29, declined steadily to 100.5° on Feb. 2. The range of temperature on the days of the calorimeter observations were: 102.0° to 102.4° on Jan. 31, and from 100.2° to 100.6° on Feb. 1. No glucose was found in the urine at any time. Examination of the blood on Feb. 1 showed 150 mg. of sugar per 100 cc. and a carbon dioxide-combining power of 50 vol. per cent.

Case 4.—Alexander De V. Empyema following lobar pneumonia. Fruit dealer, born in Italy in July, 1876. Height 168.3 cm. He was admitted to the hospital on Jan. 27, 1924, and a diagnosis of lobar pneumonia was made. On Feb. 11 signs of fluid were noted in the left pleural cavity, although the fluid could not be located by aspiration. On Feb. 15 the patient was transferred to the metabolism ward. On Feb. 20, 1,400 cc. of purulent fluid were removed from the left pleural cavity. The temperature averaged about 102° in the metabolism ward. On the days of the calorimeter observations the maximum range of temperature was as follows: 101.4° to 102.8° on Feb. 18, and 101.6° to 102.2° on Feb. 19. He was transferred to the surgical wards and operated on immediately. On return to the metabolism ward on Apr. 13 to 14 his general condition was excellent, and he had no fever. He had no glucose in the urine while under observation. The highest blood sugar was 172 mg. on the afternoon of Feb. 19, and the lowest CO₂-combining power was 57 on the previous day. The highest excretion of acetone bodies was 1.43 gm. on Feb. 17, on a carbohydrate-free diet.

RESULTS.

The object of the work was to determine whether the oxidation of a given proportion of glucose is as effective in preventing ketonuria in the presence of infection as it is in its absence. In non-infected individuals, diabetic or otherwise, ketosis appears when the glucose in the metabolism falls below a certain propor-

TABLE II.
Infection and the Ketogenic Balance.

Name. Diagnosis. (1)	Date. (2)	Metabolized per hr.						Urine.		R.Q. (11)	Rectal temper- ature. (12)	Blood.		
		Protein. (3)	Fat. (4)	Carbo- hydrate. (5)	Calories. (6)	Fatty acid. (7)	Glucose. (8)	Fatty acid: glucose as (9)	Acetone bodies per hr.* as acetone. (10)			Sugar. (13)	CO ₂ (14)	
	1923	gm.	gm.	gm.		gm	gm.				°F.	mg. per 100 cc.		
John H. Diabetes. Bronchitis.	Dec. 14 1924	2.40	6.76	0.46	74.6	7.18	2.53	2.8	0.03	0.727	100.8	375		65.3
Ellen S. Diabetes. Infected gangrene.	Feb. 4	3.89	4.65	0.88	62.8	5.98	3.60	1.7	0.05	0.748	100.9	466		
John B. Infected skin.	" 1	3.15	7.02	2.32	87.7	7.76	4.84	1.6	0.01	0.751	100.6	150		50
Alex De V. Empyema.	" 18 " 19 Apr. 15	2.78 3.42 2.91	7.36 7.24 5.25	2.04 1.29 2.76	88.2 86.7 72.0	7.90 8.09 6.06	4.39 4.00 4.97	1.8 2.0 1.4	0.05 0.05 0.02	0.746 0.740 0.767	104.4 103.3 98.6	148 172 87	56.7 63.3 58.6	

* In using the Van Slyke method for the determination of the acetone bodies 1 mg. of precipitate was taken as the equivalent of 1.20 mg. of acetone.

tion, as indicated either by a drop in the respiratory quotient or a rise in the fatty acid-glucose ratio. The threshold of ketosis is found with a respiratory quotient of 0.76 or a fatty acid-glucose ratio of 1.5. Much the same statement applies to the infected individuals of Table II.

All of the quotients shown in Column 11 of Table II are below 0.76, with the exception of the last one, but in spite of this evidence that only a small proportion of carbohydrate was oxidized, the excretion of the acetone bodies remained very moderate. The range was from 0.01 to 0.05 gm. per hour, the highest figure being very little above the level of 0.042, selected for the arbitrary threshold. The same data were utilized to calculate the fatty acid-glucose ratio and here again the values were such as would be expected to produce a ketosis in non-infected individuals. The evidence from these four patients goes to show that the same depression of the quotient (or rise in the fatty acid-glucose ratio) is accompanied by the same degree of ketosis, whether infection was present or not. Certainly no increased tendency to ketosis was observed. In other words, no evidence was found to indicate that infection had any unfavorable influence on the mechanism of the ketogenic balance.

DISCUSSION.

In observation with four infected patients it was found that the ketogenic balance, as calculated from the respiratory exchange, bore the same relation to the excretion of the acetone bodies as in previous studies on non-infected individuals. Infection did not increase the sensitiveness of our patients to the reduction in the proportion of glucose oxidized. As has been mentioned, the observations of Wilder and Winter (2) are not in accord with this conclusion. With two diabetic patients who had mild afebrile infections they found more excretion of acetone bodies than they expected from the ketogenic balance. An earlier paper from this laboratory (5) gave the erroneous impression that these investigators employed the diet as a basis for their work, rather than the foodstuffs metabolized. As a matter of fact the diet entered into their calculations only in the assumption that under the restricted conditions of the experiment the carbohydrate ingested was the same as that oxidized. On the basis of this assumption, together

with the nitrogen of the urine and the basal metabolism, they calculated the quantities of protein, fat, and carbohydrate in the metabolism and thereby the ketogenic balance. They did not consider the respiratory quotient to be reliable for short periods and did not employ it. Yet it does give the only direct measure so far devised of the oxidation of fat and carbohydrate and should give a correct picture of the metabolism when measured over a period of 3 hours. This is assuming, of course, that the quotient is not appreciably affected by disturbing factors, discussion of which will be found in other papers from this laboratory (8, 9). Although it is much to be regretted that the paper of Wilder and Winter was inaccurately quoted, there remains a distinct difference in principle between the two methods.

SUMMARY AND CONCLUSIONS.

1. The ketogenic balance was determined in relation to the excretion of the acetone bodies in the case of four patients who were suffering from infection, two of them being diabetic and the other two not.

2. These infected patients failed to excrete more acetone bodies for a given fatty acid-glucose ratio than did the non-infected individuals previously studied.

3. The evidence from our data is, therefore, that the ill effects of infection in diabetes are due, not to a disturbance in the ketogenic balance, but to an actual reduction in the amount or proportion of glucose oxidized.

In a personal communication Wilder has pointed out that in none of these experiments does the excretion of acetone bodies correspond to the excess of ketogenic over antiketogenic molecules which would be expected if the threshold ratio were unity, as previously stated (5). The exact basis of the ketogenic formula is, however, uncertain at present, and we are personally inclined to attach more importance to the association of a given respiratory quotient (or ketogenic ratio) with a given degree of ketosis, in controls and in patients with infection, than to the exact quantitative relation between theory and fact.

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AN APPARATUS FOR MEASURING THE OXYGEN CONSUMPTION OF TISSUES.*

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Various methods have recently been described for the measurement of oxidation in tissue suspensions and cultures. The three methods most commonly used are: the direct volumetric measurement of the oxygen consumed, the color changes in an added substance capable of being simultaneously oxidized or reduced, as the methylene blue technique of Thunberg,¹ and the measurement of the CO₂ evolved.

A little confusion has occasionally resulted in the assumption that these various methods are necessarily a measure of the same process or processes involved in oxidation. In incomplete oxidation which usually occurs in traumatized tissue, the oxygen consumed and CO₂ evolved may, of course, result in a respiratory quotient greatly different from one. Fleisch² has recently demonstrated that the oxygen consumption of a tissue may vary greatly both in magnitude and rate from the simultaneous reduction of methylene blue.

Of the different methods for the direct measurement of the consumption of oxygen during oxidation, the Barcroft differential apparatus has been most widely used. Warburg³ has slightly modified the Barcroft apparatus and used it extensively in his work.

* This paper is No. 41 of a series of studies in Metabolism from the Harvard Medical School and allied hospitals. The expenses of this investigation have been defrayed in part by a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

¹ Thunberg, T., *Skand. Arch. Physiol.*, 1920, xl, 1.

² Fleisch, A., *Biochem. J.*, 1924, xviii, 294.

³ Warburg, O., *Biochem. Z.*, 1923, cxlii, 70.

In fresh tissue studies it is desirable to use fairly large quantities of material so as to obtain more uniform samples, to reduce the error resulting in the rapid weighing of moist, finely cut tissue, and to increase the oxygen consumption to magnitudes more easily and accurately measured. The difficulty in a larger apparatus of the Barcroft type is that the CO_2 does not diffuse rapidly

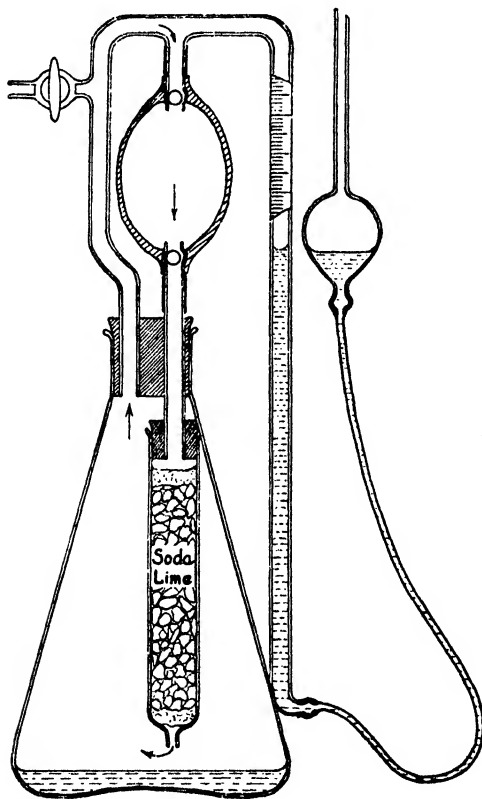


FIG. 1.

enough to be completely absorbed by the alkali. Another difficulty of the Barcroft apparatus is that the consumption of oxygen decreases its tension within the flask.

The type of apparatus we have found successful is shown in Fig. 1. An Erlenmeyer type flask is used as the receptacle for the material to be oxidized, because of the large surface exposed for

oxygen consumption. A slight shaking motion imparted to the flask keeps the tissue suspension sufficiently saturated with the oxygen at the tension within the flask so that this does not become a limiting factor. The oxygen consumed is measured in the leveling manometer at the side. The CO_2 liberated is absorbed by the soda-lime tube through which the air is circulated by means of the rubber bulb fitted with valves at the top of the flask. The rubber bulb maintains a surprisingly constant volume after the original stiffness has once been removed by use. The bulb, however, must be of a type with fairly heavy walls and rubber of good elasticity. For highly accurate work we have substituted a small specially constituted rotary gear pump in place of the bulb.

In use the apparatus is placed either singly or in groups, mounted in a frame in a constant temperature water or air bath. We usually have used a 500 or 700 cc. flask and 15 to 30 gm. of finely cut fresh tissue suspended in about an equal amount of Ringer's solution or blood plasma. The stop-cock at the top of the apparatus is opened to the outside air and the liquid in the manometer burette set at a low level. After temperature equilibrium has been obtained (about 10 minutes in a water bath as determined by a blank), the stop-cock is closed, the CO_2 absorbed by about 20 compressions of the bulb, and the original volume observed. Clove oil may be used in the manometer, but we have found distilled water to which a few drops of 0.01 N NaOH and phenolphthalein have been added quite satisfactory. The alkalinity of the solution decreases the surface tension of the meniscus. Check readings on the burette must be taken by oscillating the meniscus by means of moving the leveling bulb and noting whether it comes to rest at the same value as before. This important check in burettes of small diameter is not possible in the differential type of manometer.

THE INFLUENCE OF THE TENSION OF MOLECULAR OXYGEN UPON CERTAIN OXIDATIONS OF HEMOGLOBIN.

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INTRODUCTION.

The disputed interrelations of HbO_2 , Hb, and methemoglobin have been clarified by the electrochemical studies of Conant (1) who has shown that the oxidation-reduction potential in a mixture of these three blood pigments is referable to the potential between hemoglobin and methemoglobin. Thus, methemoglobin is the oxidation product of reduced or deoxygenated hemoglobin.

As a result of these studies it seems probable that the iron of the hemoglobin molecule is in the oxidized or ferric state in methemoglobin and in the reduced or ferrous state in reduced, oxygenated, and carbon monoxide hemoglobin. Since it is convenient to have a term which embraces all of the active hemoglobin whether reduced or in combination with oxygen or carbon monoxide, it shall be referred to here as ferrous hemoglobin.

The present paper is concerned with the influence of the tension of molecular oxygen upon the oxidation of hemoglobin to methemoglobin. It will be shown that in oxidations of the type here reported, the tension of molecular oxygen exerts two distinct influences upon the reaction. All of the oxidations studied in this paper may be grouped under the general heading of "oxygen activation." In each case, at least two reactions are involved: a preliminary reaction in which molecular oxygen unites with an autoxidizable or easily oxidized substance to form an oxidizing agent; the oxidizing agent thus formed then oxidizes the iron of hemoglobin to methemoglobin. Thus, in this type of oxidation the tension of molecular oxygen will determine not only the for-

mation of the active oxidizing agent, but will also determine the concentration of the substances actually oxidized to hemoglobin. The oxidations chosen for the illustration of these relations consist of the formation of methemoglobin by the following agents: filtered, sterile extracts of the cellular substances of pneumococci; suspensions of anaerobic bacilli; turpentine; cod liver oil; linseed oil; sterile extracts of the alcohol-soluble constituents of potato juice; and the spontaneous formation of methemoglobin in sterile, drawn blood.

Methods.

The experimental investigation of the influence of oxygen tension upon the oxidation of hemoglobin by biological agents is complicated by the difficulty of maintaining definite tensions of molecular oxygen in reaction mixtures which possess marked oxygen-consuming and reducing powers, and further by the necessity of excluding the participation of contaminating microorganisms during the reactions. These difficulties have been overcome by the combined use of chemical and bacteriological procedure. The technique employed in this laboratory (2) in physicochemical studies of gas and electrolyte equilibria in blood has been used to maintain the reaction mixtures at known tensions of molecular oxygen. Sterile reagents and the aseptic technique of the bacteriologist have been employed throughout; the absence of foreign bacteria in the experiments was proven by bacteriological tests on the reaction mixtures. The general procedure is outlined below.

Chemical.—Equal, measured amounts of blood or of hemoglobin solution were introduced into large tonometers. Each sample was then deoxygenated by evacuation and by saturation with hydrogen for 30 minutes at 38°C. Known gas mixtures calculated to give the desired tension of molecular oxygen were introduced into the tonometers containing the reduced hemoglobin. After equilibration in the water bath, the tonometers contained hemoglobin of the desired degrees of oxygen saturation corresponding to the known tension of oxygen in the gas phase. In the tables the percentage of ferrous hemoglobin in the oxygenated and deoxygenated state is given. Since these data were obtained from the oxygen dissociation curve of blood, they are to be regarded as only approximate. Equal measured amounts of the pneumococcus extract or other test substance were then introduced into each of the tonometers through a three-way stop-cock.

The reaction mixtures (test substance plus hemoglobin at different ten-

sions of molecular oxygen) were rotated in the water bath for the periods designated in the text. At the end of the reaction periods, the mixtures were cooled in ice; hemoglobin determinations were made at once by the oxygen capacity method of Van Slyke, using the technique and apparatus described by Van Slyke and Neill (3). Since the samples were cooled to 5°C. and analyzed immediately after 2 minutes saturation with air, the effect of the change in oxygen tension between the end of the reaction period and the time of actual analysis has been minimized. (Later experiments, however, indicate that carbon monoxide capacities are to be preferred to oxygen capacities in experiments of this nature.) Spectroscopic observations of the presence or absence of methemoglobin were made simultaneously with the gasometric hemoglobin analyses.

Tonometers which were relatively large (300 cc.) in comparison to the volume of the liquid mixtures (5 to 8 cc.) were used to prevent significant changes in the oxygen tension during the reaction. A constant pH of approximately 7.5 was obtained in all of the experiments by the presence of pH 7.5 phosphate at a concentration of 25 mM in all reaction mixtures (with exception of the blood used in experiments on spontaneous methemoglobin formation). The test substances used were stored in sealed containers to protect them from oxidation, previous to their introduction into the hemoglobin or blood.

Bacteriological.—All of the reagents used were sterilized either by heat or by Berkefeld filtration. The blood and hemoglobin solutions were obtained by aseptic technique and were proved sterile by subcultures.

Before each experiment the following apparatus was sterilized. Stop-cocks were removed from the tonometers and placed in Petri dishes; the open ends of the tonometer and the stop-cock openings were plugged with cotton. The tonometers and stop-cocks, together with a glass bulb stuffed with cotton, were sterilized in the oven at 150°C. A number of lengths of rubber tubing and several small capillary U-tubes were placed in Petri dishes and sterilized in the autoclave. Stop-cock grease and mineral oil were also autoclaved.

After sterilization, the cotton plug was removed from the stop-cock opening of the tonometer; the stop-cock was greased with the sterile lubricant and replaced in the tonometer. Sterile rubber tubing was placed on both ends of the tonometers. The sterile blood or hemoglobin solution was measured into the tonometer with sterile pipettes. The bulb containing the sterile cotton was attached to the gas manifold and used as a filter for the gas mixtures introduced into the tonometers.

After the evacuation and preliminary saturations of the blood or hemoglobin solutions, the rubber tubing attached to the stop-cock end of the tonometer was flamed and cut with sterile scissors, and the sterile capillary U-tube was attached. The test substance (pneumococcus extract, etc.) was introduced into the tonometer by means of a sterile pipette attached to the U-tube which was connected to the three-way stop-cock of the tonometer. By the use of this technique, it was possible to carry through without contamination the entire procedure up to the time of the actual

analyses. The absence of contaminating microorganisms was proved by subcultures of all reaction mixtures at the time of the final analyses.

Influence of the Tension of Molecular Oxygen upon Methemoglobin Formation by Sterile Pneumococcus Extracts.

The formation of methemoglobin by pneumococci when growing on media containing blood is a well known phenomenon. Sterile extracts of the cellular substances, entirely free from living or formed cells, also possess the power of oxidizing hemoglobin (4, 5, 6). The mechanism of the oxidation of hemoglobin by pneumococci has been discussed in previous papers (4, 5).

TABLE I.

Influence of Oxygen Tension Upon the Oxidation of Ferrous Hemoglobin by Sterile Pneumococcus Extract.

Tension of molecular oxygen.	Concentration of dissolved oxygen.	Proportion of ferrous hemoglobin as		Initial concentration of ferrous hemoglobin.*	Final concentration of ferrous hemoglobin.	Ferrous hemoglobin oxidized	$\text{Hb} \times \text{O}_2$ Met Hb
		HbO ₂	Hb				
mm.	mm	per cent	per cent	mm	mm	mm	
0	0.0	0	100	6.21	6.16	0.05	
10	0.0129	20	80	6.21	2.99	3.22	0.0096
20	0.0258	40	60	6.21	2.40	3.81	0.0098
35	0.0453	70	30	6.21	2.64	3.57	0.0100
140	0.181	100	0	6.21	4.76	1.45	
700	0.906	100	0	6.21	5.82	0.39	

* The value for the initial concentration of ferrous hemoglobin is the oxygen capacity of the control.

A number of experiments have been made to investigate the influence of oxygen tension upon methemoglobin formation by pneumococcus. Sterile extracts of the cellular substances have been used in place of the living cells to limit the influence of molecular oxygen to its effect upon the reaction itself, uncomplicated by the influence of oxygen upon cell growth. The protocol of a typical experiment is presented in Table I.

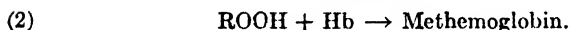
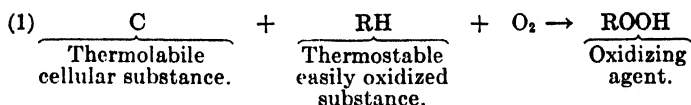
The procedure used is the same as that outlined in the description of "Methods." The tonometers were rotated in the water bath for 2 hours. The sterile pneumococcus extract was prepared by the methods previously described (7). It was

filtered through a Berkefeld candle and was proved sterile by cultural and animal tests.

The data presented in Table I reveal the following relations of oxygen tension to the oxidation of hemoglobin by sterile pneumococcus extracts. In the test in which the oxygen tension approached zero, only traces of methemoglobin were formed. Again, in the higher oxygen tensions at which most of the hemoglobin was bound as oxyhemoglobin, only a small amount of methemoglobin was formed. Evidently, the formation of methemoglobin is most marked at those oxygen tensions which permit the deoxygenation of a large amount of hemoglobin.

Since the oxygen tension influences both the formation of the oxidizing agents and the concentration of the substance which is oxidized to methemoglobin, these facts are in agreement with the proposed (4, 5) explanation of the mechanism of methemoglobin formation by pneumococcus.

The formation of methemoglobin by pneumococcus may be indicated by the following expressions.



Specifically, in the present experiments it was found that the methemoglobin formed was proportional to the concentration of reduced hemoglobin and to the tension of oxygen. This may be represented by the expression

$$[\text{Methemoglobin}] = [\text{constant}] \times [\text{reduced hemoglobin}] \times [\text{oxygen tension}]$$

or

$$(3) \quad \frac{[Hb] \times [O_2]}{[\text{Methemoglobin}]} = K$$

Although further experimental data are required for definite proof of this relation, calculations made from data already at hand indicate that Equation 3 expresses the reaction of methemoglobin formation by pneumococcus. The actual concentrations of reduced hemoglobin were not determined, but were calculated for the different oxygen tensions from the oxygen dissociation

curves of blood at the same pH. Values of K calculated from these data at 10, 20, and 35 mm. of oxygen are sufficiently constant to suggest that Equation 3 represents the reaction studied.

Influence of O₂ Tension upon Methemoglobin Formation by Anaerobic Bacilli.

The action of anaerobic bacilli upon blood pigments has been reported in a previous paper (8). The anaerobic organisms possess marked reducing powers, deoxygenating oxyhemoglobin and reducing methemoglobin with astonishing rapidity. Their reducing actions are so pronounced that the formation of significant amounts of methemoglobin is demonstrable only if the mixtures of anaerobes and hemoglobin are maintained at the optimum oxygen tension for this type of hemoglobin oxidation.

TABLE II.

Influence of Oxygen Tension upon Methemoglobin Formation by Anaerobic Bacilli.

Reaction mixture.	Tension of molecular oxygen.	Proportion of ferrous hemoglobin as		Initial concentration of ferrous hemoglobin.*	Final concentration of ferrous hemoglobin.	Ferrous hemoglobin oxidized	Spectroscopic observation of methemoglobin.
		HbO ₂	Hb				
	mm.	per cent	per cent	mm	mm	mm	
Hb + suspension of anaerobic bacilli.	0†	0	100	2.80	2.82	0.00	—
“ “	25	50	50	2.80	2.34	0.46	+
“ “	710	100	0	2.80	2.64	0.16	—

* The value for the initial concentration of ferrous hemoglobin is the oxygen capacity of the control.

† Due to impurities in the gases used in the mixtures small traces of oxygen were probably present in the tests designated in the tables as zero oxygen tension. Analyses of the gas mixtures showed the actual oxygen tensions to be less than 3 mm.

The following experiments are similar to the preceding ones with the exception that anaerobic bacilli have been used as the hemoglobin-oxidizing agents. The results of a typical experiment with the anaerobic bacteria are given in Table II.

The procedure was the same as that previously described. Suspensions of anaerobic bacilli (*B. histolyticus*) were obtained by centrifuging broth cultures of young cells. The cell suspensions were protected from air up to the time they were introduced into the tonometers. The tonometers were rotated in the water bath for 2 hours.

In the determination of the hemoglobin by oxygen capacity, vigorous aeration of the thoroughly chilled samples and their rapid introduction into the analysis pipette of the gas machine were necessary to avoid deoxygenation of the samples due to the marked oxygen-consuming powers of the cells.

It is evident from the results of these experiments (Table II) that the tension of molecular oxygen influences the oxidation of hemoglobin by anaerobic bacilli in the same manner as that previously manifested in methemoglobin formation by pneumococci. The tension of molecular oxygen must be sufficiently high to mask the potential reducing powers of the cells and to permit the formation of oxidizing agents. The formation of methemoglobin, however, proceeds to best advantage if the oxygen tension is not too high to permit about half of the ferrous hemoglobin to be in the deoxygenated state.

Influence of O₂ Tension upon the Formation of Methemoglobin by Autoxidizable Substances.

In the preceding experiments, the hemoglobin-oxidizing systems included thermolabile cellular substances probably of ferment nature (4, 5, 8). However, during the pure *autoxidation* of certain substances, oxidizing agents are formed which oxidize hemoglobin to methemoglobin (8). The formation of the oxidizing agents by union of molecular oxygen with the autoxidizable substances is quite independent of ferment participation.

A number of substances of autoxidizable nature, such as cod liver oil, linseed oil, and turpentine, were chosen for the investigation of the influence of oxygen tension upon the formation of methemoglobin by autoxidizable substances. In addition to the above liquids, which are known to contain unsaturated substances, similar experiments have been made with phosphate solution emulsions of the alcohol-soluble substances of potato juice.

Results of experiments with cod liver oil, linseed oil, turpentine, and the alcohol-soluble substances of potato juice are presented in a joint protocol (Table III).

The alcohol extracts of potato juice were prepared as described in a previous paper (8). The turpentine, cod liver oil, and linseed oil were

fresh and were stored in the ice box in the dark until used. All were protected from oxidation until introduced into the test systems. The tonometers were rotated for 2 hours in the 38° water bath.

The hemoglobin analyses of the experiments with cod liver oil, turpentine, and linseed oil, were made on samples of the hemoglobin separated as completely as possible from the partially dried oils. By repeated separation of the chilled mixtures, samples fairly free from the oils were obtained. However, due to the difficulty of obtaining representative samples, the usual accuracy of the hemoglobin analyses was not obtained in the experiments with the oils.

TABLE III.

Influence of Oxygen Tension upon the Formation of Methemoglobin by Autoxidizable Substances.

Reaction mixture.	Tension of molecular oxygen.	Proportion of ferrous hemoglobin as		Initial concentration of ferrous hemoglobin.*	Final concentration of ferrous hemoglobin.	Ferrous hemoglobin oxidized.	Spectroscopic observation of methemoglobin.
		HbO ₂	Hb				
	mm.	per cent	per cent	mm	mm	mm	
Turpentine + Hb.	0†	0	100	4.4	4.2	0.2	—
“ + “	25	50	50	4.4	3.4	1.0	++
“ + “	710	100	0	4.4	4.0	0.4	±
Linseed oil + Hb.	0	0	100	4.4	4.4	0.0	—
“ “ + “	25	50	50	4.4	3.3	1.1	++
“ “ + “	710	100	0	4.4	3.9	0.5	±
Alcohol-soluble substance of potato juice.	0	0	100	3.4	3.2	0.2	—
“ “	25	50	50	3.4	1.4	2.0	++
“ “	710	100	0	3.4	3.0	0.4	+

* The value for the initial concentration of ferrous hemoglobin is the oxygen capacity of the control.

† Due to impurities in the gases used in the mixtures small traces of oxygen were probably present in the tests designated in the tables as zero oxygen tension. Analyses of the gas mixtures showed the actual oxygen tensions to be less than 3 mm.

It is evident from these experiments that the oxidation of hemoglobin by the oxidizing agents formed from these autoxidizable substances is a reaction similar to the formation of methemoglobin by pneumococcus. Here, again, the presence of molecular oxygen is essential for the formation of the oxidizing agent, although the oxidation of the hemoglobin is most complete at

oxygen tensions sufficiently low to permit about half of the ferrous hemoglobin to be in the deoxygenated state. An essential difference obtains, however, in the fact that thermolabile cellular substances are involved in methemoglobin formation by pneumococci (4, 5), while the oxidation of hemoglobin by the autoxidizable substances is quite independent of ferment participation.

A further point of resemblance in the action of the alcohol-soluble substances of potato juice and of pneumococci consists in the fact that both the alcohol-soluble plant constituents and the bacterial substances possess reducing, as well as oxidizing, powers (9). At the requisite oxygen tension, both form oxidizing agents which oxidize hemoglobin to methemoglobin, while in the absence of molecular oxygen, both reduce methemoglobin to hemoglobin. Thus, the oxygen tension determines not only the extent but also the direction of the reaction of these substances upon the blood pigments.

Influence of Oxygen Tension upon the Spontaneous Formation of Methemoglobin in Sterile Blood.

The apparently spontaneous conversion of hemoglobin to methemoglobin in drawn blood and hemoglobin solutions is a well known phenomenon which was observed in the early work on the blood pigments. Although certain bacteria can oxidize hemoglobin to methemoglobin, the spontaneous formation of methemoglobin observed by the chemist is probably only rarely due to bacterial action. Instead, there is considerable reason to believe that the phenomenon represents an oxidation in which the hemoglobin is oxidized by oxidizing agents, formed from unsaturated autoxidizable blood constituents or an autoxidation of the hemoglobin itself (9).

Experiments have been made to determine the influence of oxygen tension upon spontaneous formation of methemoglobin in sterile blood.

Defibrinated fresh horse blood was laked by repeated freezings and thawings. The blood was drawn with aseptic technique and maintained sterile throughout the experiment. The procedure was the same as in the preceding experiments save that the tonometers were rotated for 48 hours at 38°. Sterility was proved

by subcultures of the samples analyzed at the end of the experiment.

A preceding study (9) had revealed the fact that spontaneous methemoglobin formation could be prevented if blood or hemoglobin solutions were stored in the presence of the strong reducing conditions provided by bacterial cells. Accordingly, to one of the tonometers containing reduced hemoglobin at approximately zero oxygen tension, a measured amount of a suspension of anaerobic bacilli (*Bacillus histolyticus*) was added.

TABLE IV.

Influence of Oxygen Tension upon "Spontaneous" Methemoglobin Formation in Sterile Blood.

Reaction mixture.	Tension of molecular oxygen.	Proportion of ferrous hemoglobin as		Initial concentration of ferrous hemoglobin.*	Final concentration of ferrous hemoglobin.	Ferrous hemoglobin oxidized.	Spectroscopic observation of methemoglobin.
		HbO ₂	Hb				
	mm.	per cent	per cent	mm	mm	mm	
Sterile, laked blood.	0†	0	100	8.06	7.30	0.76	±
" " "	25	50	50	8.06	6.56	1.50	++
" " "	710	100	0	8.06	7.56	0.50	—
" " " + suspension of anaerobic bacilli.	0	0	100	6.45‡	6.47	00.0	—

* The value for the initial concentration of ferrous hemoglobin is the oxygen capacity of the control.

† Due to impurities in the gases used in the mixtures small traces of oxygen were probably present in the tests designated in the tables as zero oxygen tension. Analyses of the gas mixtures showed the actual oxygen tensions to be less than 3 mm.

‡ Calculated from the dilution of the blood and checked by colorimetric determination.

All of the tonometers were rotated in the bath for 48 hours at 38°C. Gasometric analyses of the hemoglobin were then made. The results are presented in Table IV.

The results of experiments of this nature (Table IV) show that spontaneous methemoglobin formation in sterile blood is influenced by oxygen tension in exactly the same way as is methemoglobin formation by pneumococcus. When the oxygen tension approaches zero, only a slight amount of methemoglobin

is formed. When the oxygen tension is sufficiently high to bind practically all of the hemoglobin as oxyhemoglobin, considerably less hemoglobin is oxidized than at tensions sufficiently low to permit about half of the ferrous hemoglobin to be in the de-oxygenated state.

In the sample of reduced blood which contained the anaerobic bacilli, no detectable methemoglobin formation occurred, as the blood after 48 hours at 38°C. possessed its original oxygen capacity. Hemoglobin destruction in drawn blood frequently is assigned in a casual manner to the action of bacterial contaminants. As a matter of fact, however, in systems of limited oxygen content, hemoglobin proves much more stable, in so far as conversion to methemoglobin is concerned, in the strong reducing conditions provided by bacterial cells than in sterile drawn blood or in sterile hemoglobin solutions. A previous paper (9) furnishes illustrations of the fact that the spontaneous formation of methemoglobin which occurs at 55°C. can likewise be inhibited by the reducing action of bacteria.

Influence of Carbon Monoxide upon the Oxidation of Hemoglobin by Pneumococcus Extract.

Conant has interpreted the ferricyanide reaction with oxy-hemoglobin to consist in the oxidation of the reduced hemoglobin. The preceding experiments offer considerable evidence in support of a similar interpretation of the examples of methemoglobin formation which have been studied in this paper. If high tensions of molecular oxygen inhibit the above oxidations of hemoglobin by reason of the oxygenating of the hemoglobin, relatively low tensions of carbon monoxide should likewise retard the formation of methemoglobin by binding the hemoglobin as carbon monoxide hemoglobin.

The object of the following experiment was to obtain data on the effect of CO upon the type of hemoglobin oxidations which have been studied in the preceding experiments. 3 cc. of sterile hemoglobin solution were placed in each of two tonometers. Both samples were saturated in the bath at 20 mm. tension of oxygen; 10 mm. of carbon monoxide were then added to one of the tonometers and the hemoglobin was shaken for 5 minutes to permit saturation with that tension of carbon monoxide. 0.6 cc. of sterile pneumococcus extract was added to each tonometer; this was a

relatively large amount of the bacterial extract as it was desired to make a severe test of the carbon monoxide effect. The two test mixtures were rotated in the bath at 38°C. for 1½ hours. The hemoglobin was determined by carbon monoxide capacity.

Controls were made to prove that the influence of the carbon monoxide was limited to its effect upon the dissociation of the hemoglobin. Since pneumococcus extract oxidized other substances than hemoglobin the controls consisted in proof that of these oxidations only that of hemoglobin was effected by the presence of CO. Pneumococcus extract was added to a tonometer containing the same tensions of CO and O₂ as was used in the above test mixtures. After rotation in the bath in the CO and O₂ atmosphere, tests were made of the following oxidizing properties of the extract: the formation of peroxide (7), the oxidation of pneumococcus hemotoxin

TABLE V.

Influence of Carbon Monoxide upon the Oxidation of Hemoglobin by Pneumococcus Extract.

Reaction mixture.	Gas tensions.		Proportion of the ferrous hemoglobin as			Initial concentration of ferrous hemoglobin.*	Final concentration of ferrous hemoglobin.	Ferrous hemoglobin oxidised.
	O ₂	CO	HbO ₂	HbCO	Hb			
	mm.	mm.	per cent	per cent	per cent	mm	mm	mm
Hb + pneumococcus extract.	20	0	40	0	60	5.27	0.06	5.21
“ “	20	10	0	100	0	5.27	5.22	0.05
Hb + broth.	20	10	0	100	0	5.27	5.27	0.00

* The value for the initial concentration of ferrous hemoglobin is the CO capacity of the control.

(10), and the inactivation of the thermolabile component of pneumococcus oxidation-reduction systems (11). All were found to be unaffected by the presence of the CO.

These tests proved that the formation of the active oxidizing agents and the oxidation of substances other than hemoglobin were not affected at all by the presence of CO. The controls are not included in the protocol of the experiment, which is given in Table V.

As shown in Table V, the presence of CO inhibits the oxidation of hemoglobin. In the mixture of pneumococcus extract and hemoglobin exposed to 20 mm. of O₂, practically all of the hemoglobin was oxidized to methemoglobin in the tonometer containing no CO. On the other hand, although the oxygen tension was the same in both cases, only an insignificant amount of

methemoglobin was formed when the mixture of bacterial extract and hemoglobin was exposed to 10 mm. of CO.

It seems reasonable, therefore, to infer that in the case of pneumococcus extract the CO inhibition of methemoglobin formation is due to the removal from the system of reduced hemoglobin. Since the CO does not prevent the formation of the oxidizing agents themselves, the inhibition of methemoglobin formation in the above experiment is analogous to the inhibition of methemoglobin formation at high oxygen tensions in preceding experiments. That 10 mm. of CO offer more complete protection to the hemoglobin against oxidation by pneumococcus extract than do 700 mm. of O₂, is not surprising when it is recalled that CO has about 250 times greater affinity for hemoglobin than has oxygen. That methemoglobin is formed much more slowly from HbCO than from HbO₂ has been long recognized and frequently reported (12-18).

Since reduced hemoglobin is the substance actually oxidized to methemoglobin, the real explanation of the greater difficulty of forming methemoglobin from HbCO would seem to be found in the greater difficulty of dissociating the HbCO rather than in differences in ease of oxidation of HbCO and HbO₂ themselves.

DISCUSSION AND SUMMARY.

A study has been made of the influence of the tension of molecular oxygen upon the formation of methemoglobin by certain oxidizing agents of a biological nature. All of the oxidations studied have been examples of oxygen activation in which hemoglobin has been oxidized by agents formed during the oxidation of other more easily oxidized substances. Hemoglobin oxidations of this type seem to consist of at least two reactions: (1) the formation of an oxidizing agent by union of molecular oxygen with an autoxidizable or easily oxidized substance; (2) the actual oxidation of the iron of the hemoglobin molecule by the oxidizing agent formed in the first reaction.

The tension of molecular oxygen influences each of these reactions. In the first reaction the presence of molecular oxygen is required for the formation of the oxidizing agent. In the second reaction, the tension of molecular oxygen limits the concentration of reduced hemoglobin, the substance actually oxidized to

methemoglobin. Hence, the optimum oxygen tension for the formation of methemoglobin in reactions of this type is approximately 20 mm., which permits the formation of the oxidizing agent but permits over half of the total hemoglobin to exist in the reduced form. On the other hand, in the oxidation of hemoglobin by such substances as ferricyanide, the production of methemoglobin should be most rapid at an oxygen tension of zero, since with a preformed oxidizing agent, the concentration of deoxygenated hemoglobin becomes the conditioning factor of the reaction.

The results presented in this paper are in keeping with the electrochemical findings of Conant. Evidence is presented that reduced hemoglobin is the substance oxidized to methemoglobin, and that the addition of molecular oxygen or carbon monoxide changes a hemoglobin molecule to a more difficultly oxidized substance.

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THE HEMOGLOBIN SYSTEM.

I. CLASSIFICATION OF REACTIONS.*

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WITH THE COLLABORATION OF A. V. BOCK AND H. FIELD, JR.

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(Received for publication, January 7, 1925.)

The dynamic equilibrium of hemoglobin, oxygen, acids, and bases forms the central theme of this investigation. In planning the experimental work, a thorough investigation of all the main reactions for human hemoglobin was regarded as the ideal. The present article is a brief classification of the equilibria. It would be an interesting task to retrace the steps by which our present knowledge of hemoglobin was obtained, but much of the work is so recent and accessible that a very brief introduction must suffice. The combination of the acid, carbon dioxide, with hemoglobin has been investigated by Bohr (1), Buckmaster (2), and Campbell and Poulton (3). The combination of a base with hemoglobin has been investigated by Campbell and Poulton (3), Parsons (4), and Van Slyke and his colleagues (5). The combination of oxygen with hemoglobin has been investigated by Barcroft and Hill (6). Reference should be made to the paper of Warburg (7) on CO₂, Van Slyke (8) on bases, and Brown and Hill (9) on oxygen. These give references to other work on the subject.

Prof. Henderson suggested to us the importance of working on all three reactions on the same solution of hemoglobin.

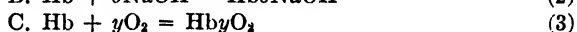
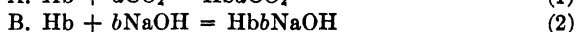
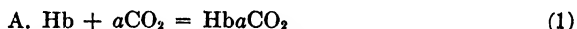
The general nature of the curves for the three main reactions can be seen in Fig. 1 of this article. The following papers will treat the three reactions individually.

* This paper is No. 42 of a series of articles on the physiology and pathology of blood from the Harvard Medical School and allied hospitals, a part of the expenses of which has been defrayed from a grant of the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

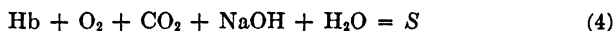
The hemoglobin system of these experiments is a simplified model of blood, with only five components, hemoglobin, oxygen, water, carbon dioxide, and sodium hydroxide.

Definitions.

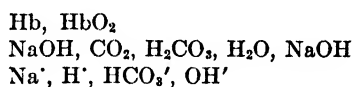
The fundamental substance is pure reduced hemoglobin, and the symbol Hb is used to denote 16,600 gm., the mass containing 1 atom of iron. The three dynamic equilibria can be written in the form:



The maximum value of y is of course 1 mol of oxygen. The maxima of a and b are not known. They exceed 4. The three reactions all influence one another; therefore, the reactions of hemoglobin must be studied in a five component system.



These components react among themselves to form a great many other substances, consequently S can be defined by stating the masses of any set of five, provided the list does not include a group like H, OH where the value of one determines the other. The more important substances are listed below.



The compounds of Hb, CO_2 , and NaOH are too indefinite to list as components. In spite of its complexity the hemoglobin system can be studied very thoroughly, because two classes of measurements can be made: first, chemical analyses, giving total masses of Hb, O_2 , CO_2 , NaOH, and H_2O ; and secondly, gas pressure and E. M. F. measurements which give the concentrations or active masses of O_2 , CO_2 , and OH. The combination of these measurements and heats of reaction encourages the hope of determining the fundamental equation of the system in the sense used by Gibbs (10).

$$U = TS - pv + \mu_1 m_1 + \mu_2 m_2 + \mu_3 m_3 + \mu_4 m_4 + \mu_5 m_5 \quad (5)$$

U = internal energy, T = temperature, S = entropy, p = pressure, v =

volume. The m terms are the masses of each component, the μ terms are the potentials of each component. The potentials can be calculated from gas pressure and E. M. F. data.

$$\mu = RT \log p + \text{constant}$$

Where the μ terms are independent of one another equation (5) is very convenient, but it would be difficult to express results in that form for all reactions where the terms are not independent. For most practical purposes, it is best to consider the fundamental reactions one at a time, choosing a different set of components for each reaction.

a , b , and y are the acid, base, and oxygen bound per mol of Hb.

These can be expressed in three equations:

$$a = f(\text{Hb}, \text{HbO}_2, \text{H}, \text{HCO}_3, \text{H}_2\text{O}) \quad (6)$$

$$b = f(\text{Hb}, \text{HbO}_2, \text{OH}, \text{Na}, \text{H}_2\text{O}) \quad (7)$$

$$y = f(\text{Hb}, \text{O}_2, \text{OH}, \text{Na}, \text{H}_2\text{O}) \quad (8)$$

TABLE I.

Three Main Reactions of Hemoglobin and Influence of a Third Component.

Reaction.	0		1	2	3	4
	Pure.		O ₂	CO ₂	NaOH	H ₂ O
Hb-CO ₂	A.	Buckmaster.	Van Slyke and Hastings.			
Hb-NaOH....	B.	Van Slyke and Hastings.				
Hb-O ₂	C.	Barcroft.		Bar- croft.	Bar- croft.	Bar- croft.

The influence of one reaction on another can be illustrated in the form of a table (Table I).

It will be seen that all the simple curves, A_0 , B_0 , C_0 , have been investigated, but of the nine surfaces, A_1 , A_3 , A_4 , B_1 , B_2 , B_4 , C_2 , C_3 , and C_4 , no less than five have never been studied. In the four actually investigated, the data are limited to two or three points.

In the practical work, our experiments are arranged under three headings.

Reaction A: The pure CO₂ curve and Δa , the difference in CO₂ bound by Hb and HbO₂ at the same tension.

Reaction B: The hemoglobin base curve and Δb at different pH levels.

Reaction C: The pure oxyhemoglobin curve and the influence of OH and H_2O .

The results obtained can be presented in a summarized form in Fig. 1.

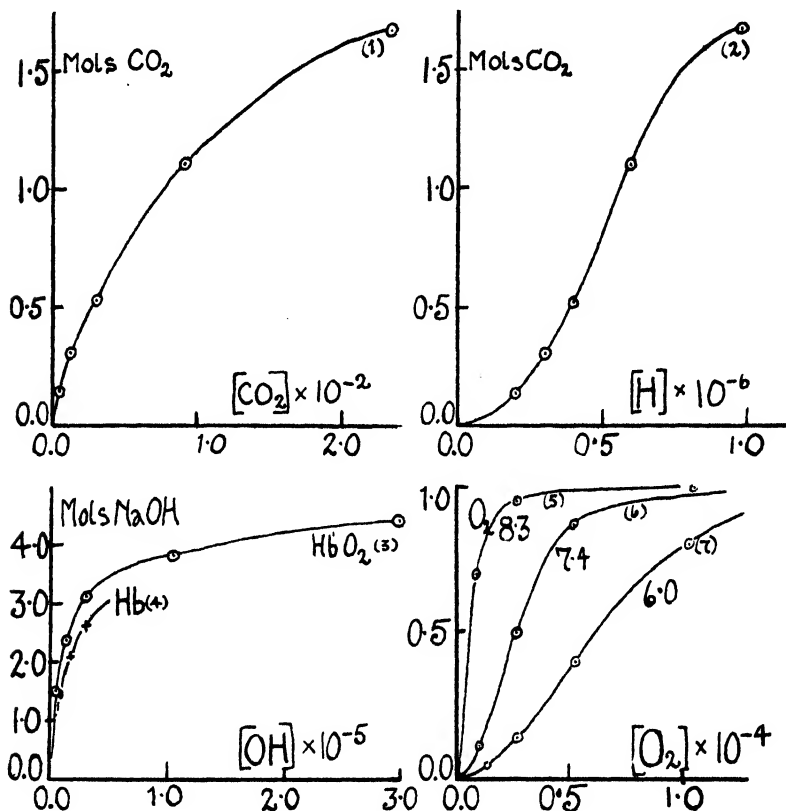


FIG. 1.

Reaction A. $Hb + CO_2$.

Curve 1 shows mols of CO₂ bound per mol Hb, plotted against concentration of dissolved CO₂.

Curve 2 shows mols of CO₂ bound, plotted against hydrogen ion concentration.

Reaction B. Hb + NaOH.

Curve 3 gives mols of base bound per mol of oxyhemoglobin, plotted against OH concentration.

Curve 4 shows mols of base bound per mol of reduced hemoglobin, plotted against OH concentration.

Reaction C. Hb + O₂.

Curves 5, 6, and 7 show mols of oxygen bound per mol of hemoglobin, plotted against oxygen concentration.

SUMMARY.

A plan of work is outlined in the dynamic equilibrium of hemoglobin with acids, bases, and oxygen, and illustrated in Fig. 1. Two new points are: (1) work on human hemoglobin; and (2) carry out all experiments on the same solution, in order to elucidate the effects of one reaction on another.

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THE HEMOGLOBIN SYSTEM.

II. THEORY OF REACTIONS WHICH DO NOT OBEY THE LAW OF CONSTANT PROPORTIONS.*

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(Received for publication, January 7, 1925.)

One of the characteristic features of the hemoglobin system is that some of the reactions contradict the well known law that all chemical reactions take place according to constant proportions by weight. The CO_2 expelled and the base bound when 1 molecule of hemoglobin is oxygenated are usually much less than 1 molecule, and the quantities vary with the conditions.

This phenomenon is explained by Prof. Hill (1) on the assumption that in each aggregate of hemoglobin particles there is one acid group which is changed on oxygenation.

One of our patients, a case of polycythemia, was examined, and it was found that the CO_2 expelled on oxygenation of his blood was practically normal, though the mass of hemoglobin was 50 per cent above the normal. This difficulty suggested the need for further examination of the problem. The importance of studying the relationship of oxygen and carbon dioxide was first pointed out by Prof. Henderson (2).

It was necessary to see if a general solution could be derived from the laws of thermodynamics.

The general criterion for chemical equilibrium was deduced by Gibbs (3).

* This paper is No. 43 of a series of articles on the physiology and pathology of blood from the Harvard Medical School and allied hospitals, a part of the expenses of which has been defrayed from a grant of the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

$$\mu_1 dm_1 + \mu_2 dm_2 + \text{etc.} = \mu_3 dm_3 + \mu_4 dm_4 + \text{etc.}$$

dm_1, dm_2 , etc. are the masses of the reactants.

dm_3, dm_4 , " " " " " " resultants.

μ_1, μ_2 are the potentials of the reactants.

μ_3, μ_4 " " " " " " resultants.

$$\mu_1 = \left(\frac{\delta U}{\delta m_1} \right) v, \eta, m_2, m_3, m_4 \text{ Gibbs' equation (104)}$$

U = the total energy of the mass.

v = volume.

η = entropy.

The integral of equation (1) is

$$\mu_1 m_1 + \mu_2 m_2 = \mu_3 m_3 + \mu_4 m_4 \quad \text{Gibbs' equation (121)}$$

The value of μ for a perfect gas is given by Gibbs' equation (285).

$$d\mu_1 = RT d \log p_1 \quad \text{Gibbs' equation (285)}$$

The equations of Gibbs stated above can be applied directly by two ordinary reactions, where m_1, m_2 , etc. are constant, but where m_1, m_2 , etc. are the functions of μ and μ_2 it is necessary to allow for this variation in the fundamental equations.

In the most important reactions, only one component fails to obey the law of constant proportions, and the equations for this special case can be derived in the simple manner given below.

Let $m_4 = f(\mu_4)$

Let m_4 be constant over the small interval $(\mu_4 + \delta = \mu_4) - \mu_4$

Since

$$\mu_1 dm_1 + \mu_2 dm_2 = \mu_3 dm_3 + \mu_4 dm_4$$

the increment of μ_4 to $\mu_4 + \delta\mu_4$ must increase μ_2 to $\mu_2 + \delta\mu_2$ if $\mu_1 dm_1$ and $\mu_3 dm_3$ remain constant.

Therefore

$$\mu_1 dm_1 + (\mu_2 + \delta\mu_2)dm_2 = \mu_3 dm_3 + (\mu_4 + \delta\mu_4)dm_4$$

$$\delta\mu_2 dm_2 = \delta\mu_4 dm_4$$

$$\frac{d\mu_2}{d\mu_4} = \frac{dm_4}{dm_2} \quad (1)$$

$$\frac{R_2 T d \log p_2}{R_4 T d \log p_4} = \frac{dm_4}{dm_2} \quad (2)$$

If m_1 is the mass of Hb or reduced hemoglobin, m_2 is the mass of oxygen.

m_3 is the mass of HbO_2 or oxyhemoglobin.

m_4 is the mass of CO_2 .

Z = mols of CO_2 expelled per mol of Hb oxygenated when the oxygen saturation is constant and the CO_2 pressure is constant.

Then the equilibrium equation can be written

$$\frac{d \log p_{\text{O}_2}}{d \log p_{\text{CO}_2}} = Z \quad (3)$$

$$\log \text{O}_2 = \int_0^{p_{\text{CO}_2}} Z d \log p_{\text{CO}_2} + K \quad (4)$$

K is the integration constant, which cannot be deduced from thermodynamics alone. Z is about 0.35 in normal blood, and about 0.25 in the case suffering from polycythemia. Preliminary experiments on the relation of $\frac{d \log p_{\text{O}_2}}{d \log p_{\text{CO}_2}}$ per constant oxygenation agreed with the values of Z determined by experiment. Formulas (3) and (4) have been deduced by a different method (Adair, 4).

The formulas given above are of interest in showing how the problems of the hospital clinic can suggest developments in quite abstract fields.

The law of mass action has been known for 50 years, but although the μ terms have been discussed by many authorities, there seems to be no thermodynamic treatment of variation in the masses of reactants in a chemical equilibrium.

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THE HEMOGLOBIN SYSTEM.

III. THE EQUILIBRIUM OF HEMOGLOBIN AND CARBON DIOXIDE.*

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WITH THE COLLABORATION OF A. V. BOCK AND H. FIELD, JR.

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The equilibrium of free carbon dioxide and carbonic acid combined to hemoglobin is the subject of this article. Many observers have found that solutions of hemoglobin hold more CO_2 than can be accounted for by simple solution, and this combined CO_2 increases with the pressure. The combined CO_2 has no simple relation to the hemoglobin concentration as in the case of oxygen; therefore, the reaction must be written with an undetermined constant a to represent mols of combined CO_2 per mol of hemoglobin.

The relation of hemoglobin to CO_2 is a very controversial subject, and there are so many points of view that it would be impossible to do justice to all in this article. The paper of Warburg should be consulted (1). There are four main questions: (1) is a a constant reproducible value at constant CO_2 pressure, (2) what is the form of the a CO_2 curve; (3) what is the nature of the Hb- CO_2 compound; and (4) what is the physiological significance of Hb- CO_2 ?

1. Parsons (2) has pointed out the wide variations in a and suggests that alleged Hb- CO_2 compounds are mixtures of hemoglobin and sodium or potassium bicarbonates. The position of the Hb- CO_2 compounds is, therefore, rather uncertain.

* This paper is No. 44 of a series of articles on the physiology and pathology of blood from the Harvard Medical School and allied hospitals, a part of the expenses of which has been defrayed from a grant of the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

2. Bohr (3) and Campbell and Poulton (4) give curves, Buckmaster (5) practically a straight line.

3. Hb-CO₂ may be an adsorption compound (Bayliss, 6) or a bicarbonate of hemoglobin.

4. Prof. Buckmaster and Prof. Bayliss attach importance to Hb-CO₂ as a CO₂ carrier in the blood, but the majority of authors regard all the combined CO₂ of the blood as NaHCO₃ or KHCO₃.

The point raised by Parsons is the first to attract the purely experimental worker. Variations in a may be due to impurities or to changes in the properties of hemoglobin due to the methods used, for it is known that the oxygen-combining power is lost if hemoglobin is not treated carefully.

Cause of the Wide Variation in CO₂-Combining Power of Hemoglobin Solutions.

In solutions prepared by ourselves and previous workers, the combined CO₂ at 40 mm. may be from 30 to 263 per cent of the oxygen capacity. As Parsons suggested, the cause of this may be undialyzed base.

The reason why the base does not dialyze away was found in a series of experiments on the osmotic pressure of hemoglobin, as affected by the hydrogen ions of the outer liquid.

The slope of the pressure-H ion curve was excessively steep compared with other proteins and even a minute concentration of OH ions in the outer liquid (10^{-6}) would enable the hemoglobin particles to retain base permanently, enough to cause errors of great magnitude. Solutions A, B, C, and D in Table I illustrate this phenomenon.

Purification of Hemoglobin without Irreversible Changes in Its Properties.

Dialysis with pure water and isoelectric water having proved unsatisfactory, Prof. Henderson suggested dialysis with the outer liquid in a saturated solution of CO₂. Although efficient in removing base, the method is not without its dangers, for long treatment with saturated CO₂ may alter the properties of hemoglobin.

The method of preparation used was a modification of that described by Adair, Barcroft, and Bock (7).

About 200 cc. of blood were drawn from an arm vein and defibrinated. The blood was centrifuged three times with ice cold 0.8 per cent salt solution. The corpuscle paste was mixed with ether and centrifuged in chilled tubes. The lowest layer, consisting of hemoglobin free from stroma protein, was pipetted off.

This solution was run into 20 cc. collodion tubes and sealed to prevent endosmosis. The tubes were placed in 2 liter jars of distilled water, saturated with CO_2 at 0°C . The water was changed twice daily. Dialysis was continued from 3 to 7 days. Two failures occurred, but excluding these the preparations were exceedingly satisfactory, as shown by the observations below.

First, the color was a brilliant red, like fresh blood laked with water, very different from the dark color familiar to those who have worked with aged hemoglobin solutions. Secondly, the oxygen capacity came within 0.3 volume per cent of the total hemoglobin by Stadie's method (8). Thirdly, the CO_2 -binding power was almost the same as in dialyzed blood, indicating that the manipulations and use of ether had no serious effect on the hemoglobin. Fourthly, when the right quantity of base was added, the solution gave oxygenated and reduced CO_2 dissociation curves almost exactly the same as normal blood. These observations are strong evidence that we have obtained hemoglobin preparations without irreversible changes.

The freedom from base was tested by analyzing the ash of Solution 6. The base was equivalent to about 0.4 volume per cent of CO_2 .

CO_2 Bound per Mol of Hemoglobin in Solutions Prepared by Various Methods.

The solutions used in this work were equilibrated with CO_2 in 250 cc. Barcroft tonometers. About 2 cc. of solution were employed and the tonometers were rotated for 15 minutes at 37.5°C .

The gas phase was analyzed with a Haldane apparatus, the liquid by means of the Van Slyke constant volume apparatus. The CO_2 combined at 40 mm. was determined by interpolation from points near 40, and the results are shown in Table I. The oxygen capacities were done with the constant pressure apparatus of Van Slyke, absorbing the oxygen with pyrogallol (9).

It will be seen that the data divide themselves into two classes. In the water dialysis experiments the figures are large and irregular, in the CO_2 dialysis experiments the results are much smaller, and what is still more important, they are practically constant. Once the irregularity due to undialyzed base is eliminated, the combination of hemoglobin and CO_2 follows the law of constant proportions at a definite tension of CO_2 .

In Table II *a* are given the actual points used for the interpolations of Table I, which proved the constancy of the CO_2 bound at a given tension. Table II *a* includes data illustrating two other questions, the CO_2 dissociation curve of Hb and the effect of oxygen tension. Every determination has been recorded, including

TABLE I.
Mols of CO_2 per Mol of Hemoglobin Bound at 40 Mm. CO_2 Tension.

Solution No.	Date.	Mols of CO_2 .	Dialysis.	Method.
A		2.10	H_2O	Bohr (Nagel's Handbuch).
B	1920	0.83	"	Buckmaster.
C	1922	2.61	"	Supplied by Dr. Ferry.
D	1922	1.78	"	Authors.
1	1923	0.40	CO_2	Horse blood.
2	Feb. 6	0.34	"	3 day dialyzed blood from A. V. B.
3	" 11	0.30	"	7 " " " " H. F.
4	Apr. 11	0.35	"	Pure Hb from A. V. B.
5	" 20	0.27	"	" " " H. F.
6	May 23	0.29	"	" " " W. A.

a few accidents where repetitions showed an error of 1 to 2 volumes per cent. The data are in chronological order, and the last two preparations are the most significant. The first three are of interest mainly because they show that even the earliest CO_2 dialysis preparations are in accordance with later work.

Preparation 1. Horse hemoglobin is a very rough approximation, for the amount of "decayed" or inactivated hemoglobin was not measured.

Preparations 2 and 3, a mixture of all the blood proteins, were a bright red color, and the unmeasured inactive Hb cannot have introduced much error, but they were colloidal jellies rather than true solutions, and they were very difficult to handle.

It is remarkable how closely the CO₂ combined by these jellies agreed with that of the solutions of pure hemoglobin.

TABLE II a.

CO₂ Combined with Hemoglobin at Various Pressures: Column 5 = Mols of CO₂ per Mol of Hb.

Preparation No.	O ₂ tension.	CO ₂ tension.	CO ₂	CO ₂ combined O ₂ capacity	pH
	<i>mm.</i>	<i>mm.</i>	<i>vol. per cent</i>		
1	150	4.0	2.4	0.2	7.04
	150	43.5	7.4	0.4	6.32
	150	112.0	19.5	1.1	6.36
	0	783.0	76.5	2.2	5.78
2	150	19.3	5.9	0.26	6.72
	150	24.5	6.7	0.28	6.65
	150	32.6	7.7	0.31	6.60
	4.5	19.2	5.1	0.22	6.63
	6.0	24.6	6.5	0.27	6.56
	5.0	32.6	8.5	0.36	6.62
3	150	9.9	4.4	0.21	6.92
	150	24.1	7.3	0.32	6.71
	150	38.8	7.9	0.30	6.47
	150	56.0	10.8	0.39	6.43
	4.6	29.0	8.4	0.36	6.87
	4.2	31.7	7.7	0.31	6.58
4	26.5	42.0	11.9	0.44	6.67
	150	43.0	12.3	0.47	6.68
	150	47.4	12.1	0.41	6.61
5	150	40.1	6.9	0.17	6.23
	5.8	12.1	2.9	0.11	6.57
	5.3	37.4	7.3	0.25	6.44
	5.0	67.2	11.4	0.37	6.34
6	150	13.2	3.9	0.14	6.69
	150	43.5	9.6	0.31	6.52
	150	97.2	17.7	0.52	6.40
	150	301.5	44.0	1.11	6.23
	0	760.0	87.0	1.68	6.01

Preparations 4, 5, and 6 were a brilliant red and very fluid. The total hemoglobin was measured as well as the inactive.

There are one or two points relating to the columns in Table II *a* which require amplification.

Column 1.—The number of the solution. A reference to Table II *b* will give additional data regarding each solution.

Column 2.— O_2 tension is the partial pressure of oxygen in the gas phase, in equilibrium with the solution, measured in mm. of mercury at $37.5^\circ C$. 150 mm. signify air and full oxidation of the Hb. 4 to 6 mm. mean that the Hb is about 95 per cent reduced.

Column 3.— CO_2 tension.

Column 4.— CO_2 volume. This is the number of cc. of CO_2 reduced to N. T. P. per 100 cc. of solution. It is the sum of dissolved and combined CO_2 .

Column 5.— $\frac{CO_2 \text{ combined}}{O_2 \text{ capacity}}$ equals the mols of combined CO_2 per mol of hemoglobin. Calculated by deducting dissolved CO_2 , $0.0672 \times CO_2$ tension, from Column 4 and dividing by oxygen capacity and inactive

TABLE II *b*.

Definitions of Properties of Solutions of Table II a

HbO_2 = oxygen capacity; Hb (total) = sum of active and inactive hemoglobin. In Solution 6 the dried hemoglobin was exactly 16 gm. per 100 cc. of solution. The base present in the ash held 0.4 of a volume per cent of CO_2 .

Solution No.	Date.	HbO ₂	Hb (total).	CO ₂ at 40 mm.	Remarks.
1	1923	10.5		0.4	Horse (28 days old).
2	Feb. 9	17.7		0.34	A. V. B. Hb + proteins.
3	" 17	18.0		0.30	H. F. " + "
4	Apr. 11	26.0		0.35	A. V. B. " pure.
5	" 20	18.4	18.9	0.27	H. F. " "
6	May 23	20.6	21.4	0.29	W. A. " "

hemoglobin. The results are calculated to two places, but the last figure may require a small correction when more is known about oxyhemoglobin methods and the solubility of CO_2 .

Column 6.—The pH was calculated from the Hasselbalch formula, in the modified form

$$pH = 7 + \frac{\log (2.166 CO_2 \text{ bound})}{CO_2 \text{ tension}}$$

It should be remembered that the formula may be incorrect when applied to hemoglobin bicarbonate.

Description of Table II a.

The most important column is the fifth, $\frac{CO_2 \text{ combined}}{O_2 \text{ capacity}}$,

for this gives the number of molecules of combined CO_2 per molecule of hemoglobin. This ratio is influenced by four independent factors: (1) the tension of CO_2 , (2) the tension of oxygen, (3) the mass of water, and (4) the temperature.

Factor 1. CO_2 Tension and the pH.—This factor will be discussed more fully in a later section, but there are one or two points which will arouse the attention of the reader in the pH calculations. Some of these are greater than pH 6.7, the isoelectric point of hemoglobin, and yet there seems to be a compound of hemoglobin and CO_2 . There are two propositions, so often repeated that their hypothetical character has been almost forgotten: (a) the pH of a solution can be calculated by the Hasselbalch formula, and (b) CO_2 and other acids can combine with ampholytes only on the acid side of the isoelectric point.

TABLE III.

Hb- CO_2	$[\text{H}] \times 10^{-7}$	$[\text{HCO}_2] \times 10^{-3}$	p_{CO_2}	pH
0.14	2.0	1.34	13.2	6.69
0.31	3.0	2.94	43.5	6.52
0.52	4.0	5.00	97.2	6.40
1.11	5.9	10.70	301.5	6.23
1.68	9.8	16.10	760	6.01

These data show that (a) or (b) or perhaps both must be wrong. The problem is an interesting one, but further investigation with electrometric methods is required to clear up the difficulty.

Factor 2. Oxygen Tension.—The preparations with 150 mm. of oxygen are fully oxidized hemoglobin, those with 4 to 6 mm. are practically fully reduced. The combined CO_2 is larger in these, but the difference is near the limits of experimental error. A few figures are given below, showing the excess of CO_2 in mols: -0.04 , -0.01 , $+0.05$, $+0.05$, $+0.08$; mean $+0.03$.

CO_2 Combined by Hemoglobin in Arterial Blood.

Prof. Henderson has given an estimate of 1 to 2 cc. These figures are rather smaller than our estimate; therefore, the evidence on the subject is set forth in Table III. In view of the number of variables, it must be understood that the estimates can only be provisional.

Hb-CO₂ depends upon both H and HCO₃ and it is not correct to calculate the Hb-CO₂ of blood from the Hb-CO₂ at a similar [H] concentration in the table, for here the [HCO₃] would be very much smaller than in blood. It is probable that a better estimate could be made from the last two points, where the [HCO₃] is rather smaller than in blood, but not excessively low.

In order to make a calculation it is necessary to assume that there is a definite number of CO₂-binding groups, which are independent of one another and obey the mass law.

$$\text{Hb-CO}_2 = a = A \frac{\alpha [\text{H}]}{1 + \alpha [\text{H}]}$$

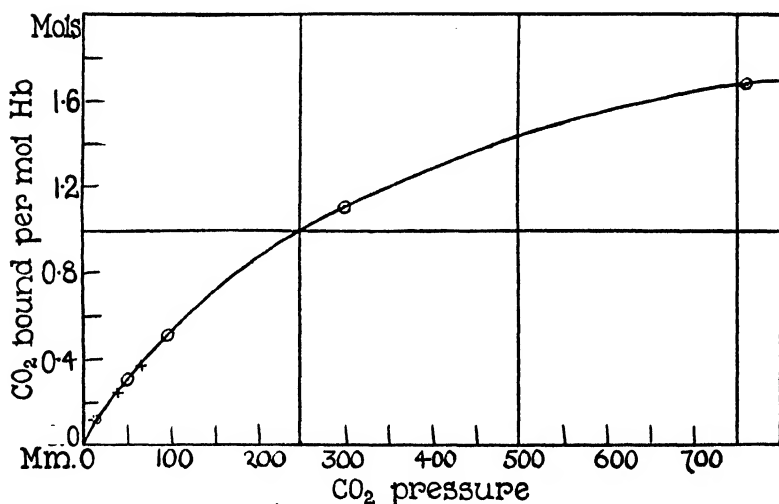


FIG. 1. Ordinates represent mols CO₂ bound per mol Hb; and abscissæ, CO₂ pressure in mm.

α is the affinity constant for [H] at constant [HCO₃], A is the number of groups.

If A is 3 and $\alpha = 0.1 \times 10^7$, the following results are obtained.

H = 5.9, Hb-CO₂ calculated = 1.11, observed 1.11

" = 9.8, " " = 1.49, " 1.68

Hence it is probable that at the [HCO₃] of blood the Hb-CO₂ will exceed the value given by the formula.

$$a = 3 \frac{0.1 [\text{H}] \times 10^7}{1 + 0.1 [\text{H}] \times 10^7}$$

Form of the Hb-CO₂ Dissociation Curve.

Fig. 1 shows the number of molecules of CO₂ bound per molecule of hemoglobin at different tensions of CO₂. The form of the curve is totally different from the oxygen dissociation curve.

First, the molecule of Hb can combine with more than 1 molecule of CO₂. Secondly, the curve is of the parabolic type, and in fact it can be fitted fairly closely by the Freundlich adsorption formula with the following empirical constants.

$$\text{CO}_2 \text{ mols bound} = 0.026 (\text{CO}_2 \text{ tension})^{0.646}$$

We have previously made the point that there is some anomaly in the pH calculations.

These three arguments favor Bayliss' (6) theory that CO₂ forms an adsorption compound with hemoglobin. Unfortunately

TABLE IV.

Source of blood.....	Horse.	A.V.B.	H.F.
Oxygen capacity per 100 cc. solution.....	20	26	29.5
Observed osmotic pressure, mm. Hg at 0°C..	232	413	650
Pressure due to hemoglobin.....	70	95	150
“ “ “ HCO ₃ ions.....	162	317	500
CO ₂ , cc. per cc. H ₂ O, inside.....	1.05	1.26	1.57
“ “ “ “ “ outside.....	0.73	0.58	0.78
Difference.....	0.32	0.68	0.79
Theoretical pressure of difference.....	243	515	600
Ionization = $\frac{p \text{ HCO}_3}{p \text{ difference}}$, per cent.....	67	62	81
Mols CO ₂ per mol Hb.....	1.4	2.2	2.2

it is impossible to be quite certain of the type of combination by studies on the form of the curve, and it was decided to continue the attack on another line by measuring the membrane equilibrium of hemoglobin and CO₂.

Membrane Equilibrium of Hemoglobin and CO₂.

If CO₂ is adsorbed on the surface of the hemoglobin molecule, it will exert no osmotic pressure. If hemoglobin bicarbonate is formed the HCO₃ will be largely ionized, and the total osmotic pressure will be the sum exerted by four types of particles: (1)

hemoglobin, (2) HCO_3 ions, (3) free H_2CO_3 , and (4) free H. The osmotic pressure measured by a collodion membrane will eliminate (3), for the pressures will be the same on both sides, inner and outer. (4) is too small to measure. Since the pressure due to (1) alone has been measured independently by one of the authors, the osmotic pressure due to HCO_3 can be readily calculated.

Measurements of the concentration of CO_2 in inner and outer liquids will give the total combined CO_2 in the inner liquid, and the osmotic pressure of the excess of CO_2 can be calculated.

The data are summarized in Table IV, and the experimental methods will be described in another paper; in this, the results alone are required.

The exact figures must be accepted with some reserve for this method of measuring ionization is a new one, and time will be required to weed out errors, but it is clear that most of the bound HCO_3 is dissociated, and it seems quite probable that all the bound CO_2 is in the form of bicarbonate.

SUMMARY.

Experiments confirmed Parson's conclusion that the wide range recorded in the quantity of CO_2 combined to hemoglobin is due to undialyzed base.

A method is described for preparing pure human hemoglobin, giving reproducible values for the combined CO_2 . Reckoned in mols per mol of hemoglobin at a pressure of 40 mm., $\text{Hb-CO}_2 = 32$ mols CO_2 , $\text{HbO}_2 = 0.29$ mol. The maximum combined CO_2 is not less than 3 mols. The CO_2 combination curve is of the parabolic type. In arterial blood about 2.8 cc. of CO_2 are combined to hemoglobin, assuming the mass law applies to the system.

The study of the osmotic pressure and membrane equilibrium of hemoglobin and CO_2 indicated that the compound $\text{Hb}(\text{CO}_2)_2$ is a bicarbonate, 70 per cent ionized. The osmotic pressure, 650 mm. of Hg, is exceptionally high if not a record for colloidal solutions.

The pH inside the corpuscle may be from 7.25 to 7.35. At 7.3, $\text{H} = 0.5 \times 10^{-7}$ and $a = 0.143$. A blood of 20 volumes per cent oxygen should have 2.86 cc. of CO_2 combined to hemoglobin. In very severe acidosis, the CO_2 bound to hemoglobin might rise to 5 cc., an appreciable fraction of the total CO_2 carried.

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THE HEMOGLOBIN SYSTEM.

IV. THE REPRODUCTION OF THE CARBON DIOXIDE CURVES OF BLOOD WITH AN ARTIFICIAL MIXTURE OF HEMOGLOBIN AND SODIUM BICARBONATE.*

By G. S. ADAIR.

WITH THE COLLABORATION OF A. V. BOCK AND H. FIELD, JR.

(From the Medical Laboratories of the Massachusetts General Hospital, Boston.)

(Received for publication, January 7, 1925.)

In the course of our studies on the equilibrium of hemoglobin, carbon dioxide, and bases, one mixture was found which, as far as the CO₂ curves were concerned, was an almost perfect imitation of blood.

TABLE I.
Co₂ Dissociation Curves of Blood and Hemoglobin.

CO ₂ pressure.	Blood oxygenated.	Blood reduced.	Hb oxygenated.	Hb reduced.
<i>mm. Hg</i>	<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>	<i>vol. per cent</i>
20	36.6	42.5	36.5	43.0
30	42.9	49.3	42.4	48.7
40	47.9	54.3	47.0	53.2
50	52.1	58.7	51.0	57.0
60	56.2	62.8	54.2	60.5
70	59.9	66.5	57.5	63.5

The figures are given in Table I.

The blood data are for the fully oxygenated and fully reduced blood of A. V. B., with an oxygen capacity of 20 = 0.00893 mol of Hb. The hemoglobin data are for a mixture of pure human hemoglobin of 18.9 volumes per cent of O₂ capacity, 0.00843 mol

* This paper is No. 45 of a series of articles on the physiology and pathology of blood from the Harvard Medical School and allied hospitals, a part of the expenses of which has been defrayed from a grant of the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

of Hb + 0.0434 mol of NaHCO_3 . The reduced curve was executed in nitrogen- CO_2 mixtures with 4 mm. of oxygen tension.

The blood curve is a little steeper than the hemoglobin curve, an effect due to the buffer action of plasma proteins, but the simple hemoglobin preparation is an astonishingly good model of the complete system.

The difference between the true and the "artificial" blood is within the normal limits of variation. The pure hemoglobin had about 7 cc. of CO_2 at 40 mm., but the addition of the right amount of base brings it back practically to the same amount as for normal blood.

This observation is of considerable importance. Many physiologists have discussed the difficult question of whether hemoglobin preparations can give reliable information as to the behavior of hemoglobin in the red corpuscle. All operations may change the product, and it is very difficult to answer this criticism.

In Paper I of this series, the point was made that hemoglobin should be regarded as part of a system of three dynamic equilibria. The operations of preparing and purifying hemoglobin alter masses of the components of the system, but they do not alter the fundamental character of the hemoglobin, for when the bases and CO_2 are replaced, the hemoglobin behaves essentially as in blood. The conception of hemoglobin as a part of a *system* is of more practical value than the usual conception of it as a very labile or unstable *substance*.

It is not denied that the hemoglobin itself may be denatured or inactivated, under certain conditions, but these changes can be avoided by careful methods of preparation.

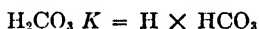
THE HEMOGLOBIN SYSTEM.
V. THE RELATION OF HEMOGLOBIN AND BASES.*

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(Received for publication, January 7, 1925.)

The subject of this article is the combination of human hemoglobin and oxyhemoglobin with sodium hydroxide, in the five component system Hb, O₂, CO₂, NaOH, H₂O. The reaction on which this work is based is quite well known (1, 2, 3, 4). A mixture of hemoglobin and sodium hydroxide exposed to constant CO₂ pressure combines with far less CO₂ than the NaOH alone; therefore, the hemoglobin must have combined with the base. The relation of H₂CO₃ and HCO₃ ions is measured; therefore, the H or OH ion of the solution can be calculated by the law of mass action, since



Van Slyke and his associates (4) have shown that the combination of these calculations gives a titration curve more accurate than the hydrogen electrode.

In the planning of this work we are indebted to Prof. Henderson and also Dr. Van Slyke whose suggestions enabled us to avoid needless duplication.

The new points in this work are: (1) human hemoglobin was used, and the figures compared with Van Slyke's data on horse hemoglobin; (2) a special method of preparation, giving hemoglobin in a very "natural" condition; (3) measurements of base bound and buffer value were carried out over a wide range of pH, in order to obtain data required for the next paper on oxygen curves;

* This paper is No. 46 of a series of articles on the physiology and pathology of blood from the Harvard Medical School and allied hospitals, a part of the expenses of which has been defrayed from a grant of the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

and (4) the difference between true base bound and the apparent value is defined and roughly estimated.

TABLE I.

Definitions of Hemoglobin Solution.

1. A. V. B. } Initials which signify blood was drawn from Doctors
2. H. F. } = A. V. B., H. F., and W. A.
3. W. A. }
4. Hb + pr. = Dialyzed whole blood, hemoglobin, and proteins.
5. Hb-CO₂ (40) = Molecules of combined CO₂ per mol of Hb at 40 mm.
6. x_{50} = Oxygen tension of half saturation.
7. B Hb (7.4) = Mols of base absorbed by preparation at pH 7.4 (affected by impurities).

Data on a Preparation.

8. O = Oxygen capacity, vols. per cent.
9. B. A. = Base added, vols. per cent CO₂.
10. O₂*t* or *x* = Oxygen tension, mm. of Hg.
11. CO₂*t* = CO₂ tension, mm. of Hg.
12. O₂*v* = Total oxygen dissolved and combined, vols. per cent.
13. CO₂*v* = Total CO₂, vols. per cent.
14. HbO₂ = Combined oxygen, vols. per cent = O₂*v* - 0.0027 O₂*t*.
15. CO₂C = Combined CO₂, vols. per cent = CO₂*v* - 0.0672 CO₂*t*.
16. B = Base bound = B. A. - CO₂C.
17. B Hb = Base bound per mol of Hb = $\frac{B}{O}$.
18. B Hb' = Base bound corrected by No. 7.
19. *y* = Percentage of hemoglobin oxygenated.
20. A = Air.
21. pH = $-\log [H] = 7 + \log 2.166 \times \frac{CO_2C}{CO_2t}$.
22. OH = Anti log (pH - 13.475).
23. Na in millimols - B. A. vols. per cent $\times \frac{1}{2.24}$.

In calculating B Hb and B HbO₂ the inactive hemoglobin is reckoned in with the Hb or HbO₂.

The method of preparation described in Paper III of this series was very rapid, giving a very natural hemoglobin at the cost of little difficulty in the estimates of certain constants.

Definitions of Terms.

The experimental measurements made are the pressures of oxygen and carbon dioxide in the gas phase and the volumes in cc. per 100 cc. of solution of CO₂ and O₂ in the liquid phase.

It is necessary to undertake a good many calculations in order to pass from these quantities to a system of units with a simple chemical interpretation. Many of the calculations are provisional, so it is necessary to give mathematical definitions of

TABLE II.

Base Bound by Pure Human Hemoglobin.

Preparation No. 5. Blood from Dr. H. F. Apr. 22.

Oxygen capacity = 18.3, total Hb (Stadie method) = 18.9.

CO₂ bound at 40 mm. Hg = 0.27 mols.

O₂ at $y = 50$ per cent = 44 mm.

BHbO₂ at pH 7.4 = 2.8.

Impurity (?) 0.0084 M HCl.

B. A.	CO ₂ t	CO ₂ o	CO ₂ C	pH	B Hb*	O ₂ t
0	12.1	2.9	2.1	6.57	0.11	5.8
	37.4	7.3	4.8	6.44	0.25	5.3
	67.2	11.4	6.9	6.34	0.37	5.0
	40.1	6.9	4.2	6.35	0.22	A.
55.2	20.8	16.6	14.2	7.17	2.18	A.
	43.5	25.5	22.6	7.09	1.75	A.
	44.8	25.8	22.8	7.04	1.72	A.
	80.5	35.2	29.8	6.87	1.46	A.
	19.2	19.5	18.2	7.31	1.97	4.9
	38.3	26.8	24.2	7.14	1.64	6.6
	40.4	27.5	24.8	7.12	1.62	7.0
	44.3	29.9	26.9	7.12	1.51	5.2
	70.6	36.7	32.0	6.99	1.34	5.1
	96.6	10.8	28.1	7.75	3.60	A.
	20.1	36.7	35.3	7.58	3.24	A.
	40.6	47.1	44.4	7.37	2.76	A.
96.6	43.4	48.0	45.0	7.35	2.73	A.
	73.7	58.3	53.3	7.20	2.23	A.
	19.3	42.1	40.8	7.66	2.96	6.4
	41.0	53.6	50.9	7.43	2.42	5.5
	41.4	53.6	50.9	7.42	2.42	6.5
	69.5	63.2	58.5	7.26	2.02	5.0

In the tables B. A. = Base added. B Hb* = CO₂ bound. B Hb = Mols NaOH per mol Hb, uncorrected.

the variables. These are collected with certain other definitions in Table I.

The material in Tables II, III, and IV is very complex and more than one interpretation could be brought forward. For

the present it is best to look on the material from the graphical standpoint, and consider what curves will be of most practical value.

Eight relations are listed below.

$f_1 \text{ pH} = \frac{\delta \text{BHb}}{\delta \text{pH}}$	Buffer values β_R .
$f_2 \text{ pH} = \frac{\delta \text{BHbO}_2}{\delta \text{pH}}$	" " β_O .
$f_3 \text{ pH} = \frac{\delta \text{BHb}}{\delta y}$	Base bound on oxygenation ΔB .
$f_4 \text{ pH} = \frac{\delta \text{pH}}{\delta y}$	pH change on oxygenation.
$f_5 \text{ pH} = \text{BHb}$	Apparent base bound.
$f_6 \text{ pH} = \text{BHbO}_2$	" " "
$f_7 \text{ pH} = B_t \text{Hb}$	True value base bound.
$f_8 \text{ pH} = B_t \text{HbO}_2$	" " " "

TABLE III.

Base Bound by Pure Human Hemoglobin.

	Preparation 4. Blood of A.V.B. Apr. 11.	Preparation 6. Blood of W.A. May 23.
Oxygen capacity.....	20.8	20.6
Total Hb from dry weight $\times 1.34$		21.4
Mols CO_2 at 40 mm. Hg tension.....	0.35	0.29
O_2 at 50 per cent.....		33.0
B HbO_2 , pH 7.4.....	2.63	1.82
Impurity HCl (?).....	0.0073 N	0

B. A.	CO_2	CO_2	CO_2C	pH	B Hb	O_2
No. 4.	0.8	67.3	67.3	9.27	5.3	A.
178	15.2	94.2	93.2	8.12	4.1	A.
	47.1	112.0	108.8	7.70	3.3	A.
	92.0	127.0	120.8	7.45	2.76	A.
	144.0	138.7	129.0	7.29	2.36	A.
	41.3	116.8	114.0	7.75	3.08	6.3
	43.7	117.2	114.2	7.75	3.06	8.4
	45.7	120.6	117.5	7.74	2.91	5.9
	82.5	132.6	127.1	7.52	2.46	7.0
No. 6.	32.3	47.3	45.1	7.48	2.05	27.1
88	74.6	63.2	58.2	7.22	1.43	44.6

The first four relations are of the greatest interest because Van Slyke and his associates (4, 5) determined them for horse hemoglobin, and because they are needed to explain the oxygen dissociation curve, treated in Paper III of this series.

Group 1. Buffer Values ((f_1) and (f_2)).

The theory of buffer values was worked out by Van Slyke (6). The method of calculation is quite simple. BHb_1 is read off at

TABLE IV.
Base Bound by Proteins of Whole Blood.

Preparation No.....	C		2	3
Source of blood.....	A. V. B.		A. V. B.	H. F.
Date.....	Jan.		Feb. 9	Feb. 17
Oxygen capacity.....	14.0		17.7	18.0
Mols CO_2 at 40 mm. Hg tension.....	0.47		0.34	0.30
B HbO_2 , pH 7.4.....			2.8	3.3

B. A.	CO_2^t	CO_2^v	CO_2^c	pH	B Hb	O_2^t	O
No. C.	15.6	5.4	4.1	6.75		A.	14.0
0	35.9	8.6	6.0	6.55		A.	
	41.4	9.6	6.6	6.54		A.	
	53.3	10.8	6.9	6.44		A.	
	19.0	6.6	5.2	6.77		5	
	38.0	9.7	6.8	6.59		5	
No. 2.	34.8	22.0	19.7	7.10	2.0	A.	13.6
47.4	24.9	22.3	20.6	7.25	2.0	5	13.6
No. 3.	16.9	6.8	5.7	6.86	0.8	A.	16.3
18.8	23.3	7.1	5.5	6.71	0.8	A.	16.3
	46.9	11.9	8.8	6.61	0.6	A.	16.3
	38.5	11.8	9.2	6.71	0.6	6	16.3
	42.5	12.1	9.3	6.67	0.6	6	16.3
84.6	19.1	45.1	43.8	7.70	3.9	A.	10.6
	61.3	56.6	52.6	7.27	3.0	A.	10.6
	37.5	53.9	51.4	7.47	3.2	6	10.6

the point pH_1 , BHb_2 at the point pH_2 . Then β_R or $\frac{dB}{d\text{pH}}$, the buffer value $(\text{BHb}_2 - \text{BHb}_1) \div (\text{pH}_2 - \text{pH}_1) = \beta_R$. Generally BHb plotted against pH gives a straight line over a fairly long range, but if β_R varied rapidly, a tangent to the BHb - pH curve

was drawn and β_R calculated for a definite pH. It is possible to prove theoretically that β_R varies with the Na ion concentration, so this should be stated. A few calculations of β_{Hb} and β_{HbO_2} are tabulated in Table V.

It will be seen that the results on man at pH 7.4 are fairly close to the result of Van Slyke and his associates (4) on horse hemoglobin. The difference seems to be a real one, for two other preparations of human hemoglobin gave 2.54 at pH 7.4 and 2.39

TABLE V.
Buffer Values of Hemoglobin.

Blood.	[Na]	pH	β_O	β_R
	M			
Man.	0.025	7.1		1.97
"	0.043	7.4	2.49	2.33
"	0.080	7.9	1.9	
"	0.080	8.6	1.0	
Horse.		7.4	2.64	2.45

TABLE VI.

Solution.	pH	ΔB	CO ₂ C
Hb + pr.	6.6	0.08	5.2
" + "	6.75	0.09	6.8
" + "	6.70	0.20	9.2
" + "	6.90	0.45	15.3
Hb pure.	7.00	0.41	32.0
" "	7.40	0.51	51.0
" "	7.60	0.56	40.0
" "	7.75	0.55	114.0

at pH 7.3. The range of variation in Van Slyke's work (4) can be seen from the four determinations 2.58, 2.66, 2.68, and 2.63. The lowest β_O horse is above the highest β_O human blood value. The maximum buffer value of one monovalent acid group is 0.575. Hence the minimum number of acid groups per mol of hemoglobin is 4.60 in the horse, and 4.34 in man. The real number may be much larger than the minimum.

Group 2. Base Bound on Oxygenation (f_3).

ΔB is the base bound when hemoglobin is oxygenated at constant alkalinity (Table VI).

CO_2C = Combined CO_2 in Volumes Per Cent in the Absence of Oxygen.

The figures for combined CO_2 are given, for it seems that ΔB is a function of the salts present as well as of the pH. The figure at pH 7.4 is 0.51 ± 0.1 , considerably smaller than Van Slyke's figure for horse hemoglobin, 0.68 ± 0.1 . Our hemoglobin was prepared differently, and γ had to be calculated, but on the whole the difference seems to be a real one.

A difference like this can hardly be reconciled with any theory, for oxygenation is supposed to affect the hematin portion of the molecule, while differences from one animal to another are supposed to be in the globin portion.

Group 3. Apparent Bose Bound ((f_s) and (f_a)).

The absolute value of the titration curve of human hemoglobin is of no little interest, but unfortunately one of us had to leave the hospital and postpone the full experimental treatment of this question.

A provisional estimate may be of some service. Van Slyke (4) found the formula for base bound by hemoglobin was

$$BHbO_2 = \beta_0 (pH - a)$$

β_0 is the buffer value at pH 7.4, a is 6.6, the isoelectric point. If the data of Table II are tested, $a = 6.28$. It seems quite probable that the formula should apply to human hemoglobin, and if such a guess is correct, we must assume that 0.8 mol of chloride remained bound to the hemoglobin in the dialyzers. The method of CO_2 dialysis is subject to this source of error, if the time allowed is too short.

The corrections were estimated as follows:

Preparation 4	$BHb' = BHb - 0.8 \pm 0.5$
" 5	$BHb' = BHb - 1.0 \pm 0.5$
" 6	$BHb' = BHb + 0.1 \pm 0.5$

A wide margin of error is given, for there is no proof that the formula applies to human hemoglobin.

The values of $BHbO_2$ are given in Table VII.

Group 4. Total Base Curves ((f₇) and (f₈)).

This relation is one of the most difficult subjects for study and this article is limited to a preliminary explanation. Sørensen (7) discusses similar problems in the chapter on the capacity of ampholytes to combine with acids and bases, but his methods are so involved that they could not be applied to practical problems. The components given below reduce the labor of computation $\text{Hb (NaOH)} = f(\text{Hb, OH, Na, H}_2\text{O})$.

Instead of Hb, consider an ideal protein R , with one acid group obeying the mass law in all its equations of dissociation.



$$K_1 [R] \times [\text{OH}] = [\text{ROH}] \quad (2)$$

$$K_2 [\text{ROH}] \times [\text{Na}] = [\text{ROH Na}] \quad (3)$$

Let b = base bound = $\text{ROH} + \text{ROH Na}$

$$\text{Therefore } b = \frac{[\text{OH}] (K_1 + K_1 K_2 [\text{Na}])}{1 + [\text{OH}] (K_1 + K_1 K_2 [\text{Na}])} \quad (4)$$

$$\text{Let } \beta = K_1 + K_1 K_2 [\text{Na}]$$

$$\text{Therefore } b = \frac{\beta [\text{OH}]}{1 + \beta [\text{OH}]} \quad (5)$$

Similarly, the acid-binding equation

$$- = \frac{\alpha [\text{H}]}{1 + \alpha [\text{H}]} \quad (6)$$

The true value of base bound is obviously given by equation (1). The value of BHb calculated by deducting the 'Bicarbonate' B or combined CO_2 from the total base added gives not the true value b but some lesser amount, for unless H is infinitely small, some CO_2 must be bound to the hemoglobin.

$$\text{BHbO}_2 = \frac{\beta [\text{OH}]}{1 + \beta [\text{OH}]} - \frac{\alpha [\text{H}]}{1 + \alpha [\text{H}]} \quad (7)$$

BHbO₂ has been tabulated, and in order to determine B, HbO₂ it would be necessary to add $\frac{\alpha [\text{H}]}{1 + \alpha [\text{H}]}$ to the tabulated values.

So much speculation is involved in determining α that no estimate can be made of the probable error of the calculation.

In Paper III of this series, a formula was deduced for the ideal case with three independent acid-binding groups obeying the mass law. The acid bound was given by the formula

$$a = 3 \frac{0.1 [\text{H}] \times 10^7}{1 + 0.1 [\text{H}] \times 10^7}$$

This formula has been used to get approximate corrections for Table VII. The apparent base bound, BHbO_2 , is derived from Table II and the corrections for impurity are based on Van Slyke's formula (4). The relative values are more accurate than the absolute. The absolute error is less than ± 0.5 .

Parsons (1) has put forward the theory that the compound

TABLE VII.

Apparent Base, BHbO_2 , Total Base, B_tHbO_2 , and Mols CO_2 Bound per Mol of Human Oxyhemoglobin, a .

pH	Na	$[\text{OH}] \times 10^7$	a	BHbO_2'	$\text{B}_t\text{HbO}_2'$
6.9	0.0248	2.7	0.34	0.45	0.79
7.0	0.0248	3.3	0.27	0.70	0.97
7.2	0.0434	5.3	0.19	1.23	1.42
7.4	0.0434	8.4	0.12	1.80	1.92
7.6	0.0434	13.3	0.07	2.28	2.35
7.8	0.0795	21.1	0.04	2.70	2.74
8.0	0.0795	33.5	0.03	3.06	3.09
8.5	0.0795	105.9	0.01	3.80	3.81
9.0	0.0795	335.0	0.00	4.40	4.40

of sodium and hemoglobin obeys the mass law. This theory can be tested with the aid of the data, column $\text{B}_t\text{HbO}_2'$, assuming that there are five independent base-binding groups of equal strength.

$$\text{B}_t\text{HbO}_2' = 5 \frac{\beta [\text{OH}]}{1 + \beta [\text{OH}]}$$

The following calculations show the constancy of β .

pH.....	6.9	7.4	7.8	8.5
$\beta \times 10^{-7}$	0.067	0.074	0.057	0.030

The values of β are not absolutely constant, but in view of the many sources of error, it is fair to say that the mass law is quite

accurate enough for ordinary purposes. The isoelectric point may be calculated from the equations

$$3 \times \frac{0.1 [\text{H}] \times 10^7}{1 + 0.1 [\text{H}] \times 10^7} = 5 \times \frac{0.07 [\text{OH}] \times 10^7}{1 + 0.07 [\text{OH}] \times 10^7}$$

Since $\text{OH} = \frac{3.3}{\text{H}}$; H can be calculated as approximately 1.7; therefore, $\text{pH} = 6.77$. This is in rough agreement with the isoelectric point of 6.6 determined by electrophoresis.

Ionization of Sodium Hemoglobinate.

Let π = osmotic pressure divided by the theoretical osmotic pressure of the oxygen equivalent of hemoglobin. At pH 9.0, the value of $\text{BHB}' = 4.4$, therefore π should exceed 4.4+ per full ionization. The observed maximum π was 2.59, and deducting 0.3 per protein pressure, the ionization of the sodium hemoglobinate appears to be about 50 ± 15 per cent.

CONCLUSIONS.

Human hemoglobin has its most efficient buffer action at pH 7.4, the blood reaction. In Van Slyke's notation, $\beta_0 (\text{HbO}_2)$ is 2.47 ± 0.07 . $\beta_R (\text{Hb}) = 2.31 \pm 0.1$. The buffering effect of human hemoglobin is 7 per cent less than that of horse hemoglobin.

The base bound on oxygenation was studied over a wide range of alkalinity. ΔB rises from 0.08 at pH 6.6 to 0.51 at pH 7.4 and increases to 0.55 at pH 7.8. The figure at blood reaction is 25 per cent less than Van Slyke's figure (5) for horse hemoglobin at the same reaction.

A distinction is drawn between BHb , the apparent value of the base bound, and B_tHb the true value. The simplest mechanism which will represent the base-binding power of hemoglobin is one of five independent acid groups of equal strength the constant being about $1/17.4$ that of carbonic acid.

The formulas

$$\text{B}_t\text{HbO}_2 = 5 \times \frac{7 \times 10^8 [\text{OH}]}{1 + 7 \times 10^8 [\text{OH}]} \approx 0.5$$

$$\text{BHbO}_2 = 5 \times \frac{7 \times 10^8 [\text{OH}]}{1 + 7 \times 10^8 [\text{OH}]} - 3 \times \frac{10^8 [\text{H}]}{1 + 10^8 [\text{H}]} \approx 0.5$$

give an approximate representation of the experimental data.

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THE HEMOGLOBIN SYSTEM.

VI. THE OXYGEN DISSOCIATION CURVE OF HEMOGLOBIN.*

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WITH THE COLLABORATION OF A. V. BOCK AND H. FIELD, JR.

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(Received for publication, January 7, 1925.)

This work gives the oxygen dissociation curves of solutions previously investigated in regard to their acid-binding and base-binding properties. Table I gives oxygen dissociation curves of pure hemoglobin. These are on the lines of the work of Hüfner (1), Barcroft and Roberts (2), Adolph and Ferry (3), and Adair, Barcroft, and Bock (4), but owing to the fact that the previous work gave rough estimates of the amount of impurity in the dialyzed solution, it was possible to give a theoretical explanation of the phenomena. The tensions at half saturation in this table are interpolated.

Table II gives the relation of the affinity constant of oxyhemoglobin and the pH in the presence of sodium bicarbonate. This appears to be the first test of this relation in an homogeneous system. Blood was investigated by Barcroft and Peters (included in the report of the Monte Rosa Expedition of 1911 (5)), and Barcroft and others (6).

Table III gives dissociation data in a salt solution.

Theories of the Oxygen Dissociation Curve.

The curves of Fig. 1 show the relation between the oxygen pressure and y , the percentage of hemoglobin oxygenated in

* This paper is No. 47 of a series of articles on the physiology and pathology of blood from the Harvard Medical School and allied hospitals, a part of the expenses of which has been defrayed from a grant of the Proctor Fund of the Harvard Medical School for the study of chronic diseases.

solutions of different alkalinity. Similar families of curves have been prepared for blood by Barcroft and Poulton (7) and Bock, Field, and Adair (8).

There has been much speculation over the meaning of these curves.

We cannot attempt to do justice to all the points of view now

TABLE I.

Oxygen Dissociation Curves of Dialyzed Human Hemoglobin with Small Traces of Acid or Base. B = Acid.

Date.	Subject.	O ₂ capacity.	Base.	pH	α	ν
		vol. per cent	vol. per cent		mm. Hg	per cent saturation
May 14	H. F.	21.9	-1.7	6.0	47.7	50.0
					5.6	3.7
					11.6	7.8
					23.4	19.8
					26.8	22.0
					56.7	60.0
					71.9	73.5
					86.1	78.3
					49.6	53.2
Apr. 21	"	16.6	-1.0	6.3	44.0	50.0
" 27	"	16.6	-1.0	6.3	11.7	14.3
					14.0	16.0
					34.9	41.2
					62.6	61.5
					67.3	78.9
May 23	W. A.	20.6	+0.1	6.6	33.0	50.0
					32.9	51.6
					46.9	68.7
					51.9	73.7
" 24	"	20.6	+4.5	8.3	5.0	50.0
					8.4	76.9
					18.1	93.5

held. The original papers must be consulted. The following brief summary will show the main lines of argument.

I. Adsorption.—Ostwald (9) and Bayliss (10). Prof. Bayliss regards surface forces on the hemoglobin molecule as the most important factor and criticizes the application of the mass law.

II. Ionization.—Henderson (11), Adolph and Ferry (3), and

Parsons and Parsons (12). Prof. Henderson suggests the S-shaped curve may be due to changes in base bound on oxygenation.

III. Aggregation.—Douglas, Haldane, and Haldane (13) and Haldane (14). Reduced hemoglobin more aggregated than oxyhemoglobin.

IV. Aggregation and Osmotic Pressure.—Barcroft and Roberts (2), Hill (15), Barcroft and Hill (16), Barcroft (7), Hill (17),

TABLE II.

Influence of pH, NaHCO₃, and CO₂ on the Affinity of Hemoglobin for Oxygen.

A = log x of Acid Preparation 3 minus log x observed at the same saturation. Column 1 gives the source of the blood. CO₂ p = tension of CO₂.

Source.	A	pH	CO ₂ combined.	pCO ₂	α	γ	α_{50}
			vol. per cent	mm.	mm. Hg	per cent saturation	
F. and F.	0	6.0*	2.0	45.9	49.6	53.2	47.6
A. V. B.	0.27	6.66*	9.0	42.0	26.5	53.0	25.5
W. A.	0.21	6.98	9.0	20.2	28.7	45.7	29.3
"	0.19	7.06	19.0	35.7	49.3	75.5	30.8
H. F.	0.43	7.14	24.2	38.3	6.6	12.7	17.7
W. A.	0.31	7.22	74.6	58.2	44.6	82.8	23.3
H. F.	0.42	7.44	53.0	41.4	6.5	12.0	18.1
"	0.39	7.42	50.5	41.5	19.1	47.8	19.4
"	0.40	7.43	47.0	38.0	35.5	82.8	19.0
W. A.	0.36	7.48	45.1	32.3	27.1	76.7	20.8
A. V. B.	0.43	7.52	127.1	82.5	7.0	14.0	17.7
H. F.	0.43	7.66	40.8	19.3	6.4	16.8	17.7
A. V. B.	0.47	7.75	114.2	43.7	8.4	20.0	16.1
W. A.	0.96	8.36*	0	0	8.4	76.9	5.2
"	0.87	8.36*	0	0	18.1	93.5	6.4

The O₂ pressure at $\gamma = 50$ per cent = anti log (1.678-A).

The pH estimates starred have a range of error of ± 0.3 .

The acid preparation is the first in Table I.

Hill (18), Barcroft and others (6), and Brown and Hill (19). In distilled water, hemoglobin is unimolecular—molecular weight 16,666—and the dissociation curve is a rectangular hyperbola. In salt solutions, polymerization takes place—apparent degree of aggregation is n —where n is strictly defined by Hill as the reduction in osmotic pressure whatever the cause. This theory leads to the formula

$$y = \frac{Kx^n}{1 + Kx^n} \quad (1)$$

y = Oxygen saturation.

x = Oxygen pressure.

TABLE III.

Oxygen Dissociation Curve of Hemoglobin in Isotonic Salt Solutions of Composition 0.1 M K, 0.1 M Cl, M/15 PO₄, (M/7.5) Na, pH 8.08.

HbO₂ = oxygen capacity. x = O₂ tension. y = percentage of oxygenation. A, B, C, and D = Preparations 1 and 2 on man and 1 and 2 on horse.

	HbO ₂	x	y		HbO ₂	x	y
	vol. per cent	mm. Hg	per cent saturation		vol. per cent	mm. Hg	per cent saturation
A	22.5	9.2	32.0	B	19.6	6.6	17.6
		1.4	43.5			11.5	39.4
		11.0	48.5			12.9	48.8
		15.3	65.5			13.2	53.0
		22.7	84.9			23.2	79.7
		34.0	90.0			37.7	92.0
D	21.9	3.6	11.2	B	9.8	44.5	92.3
		12.6	53.7			12.0	49.2
		20.5	79.7			17.4	72.6
		29.4	87.5			24.3	83.0
		47.2	93.7			35.5	96.4
D	21.9	14.0	63.5	C	15.0	51.0	94.1
		20.5	80.0			5.8	36.0
D	7.3	10.1	44.1			14.8	79.0
						18.3	89.0
						42.5	98.0
D	29.3	12.9	59.8		7.5	75.0	100.0
		19.2	69.8			4.2	35.5
		21.1	77.0			13.9	80.0
		15.9	73.1			19.0	90.8
		19.7	79.8			42.7	96.0
		27.1	91.1				

There are two fundamental postulates in Prof. Hill's theory:

(1) the osmotic pressure of hemoglobin is $\frac{1}{n}$ of the equivalent of oxygen.

p :

where C = gm. Hb per 100 cc. solvent and $n = 2.2$ (2). Hemoglobin and oxyhemoglobin are homogeneous and separable substances.

Adair tested these assumptions by direct experiment. Qualitatively, Hill's theory was confirmed for n was over 1, showing that aggregates with more than 1 iron atom were present. Quantitatively, Hill's theory was not confirmed.

Testing equation (1) it was found that the relation of p and C was a curve.

$$p = \frac{10 \cdot 21}{4} \left(\frac{C}{1 - 0.015C} \right) \quad (2)$$

The apparent value of n was not constant.

Adair's Theory.

Adair's theory (20) is that the molecular weight of hemoglobin is 66,700.

Equation (2) was applied to calculate a dissociation curve, assuming homogeneous equilibrium. The formula was

$$\log x - \log x_{50} = \frac{1}{4} \log \frac{p_{\text{HbO}_2}}{p_{\text{Hb}}} + 0.000638 (p_{\text{HbO}_2} - p_{\text{Hb}}) \quad (3)$$

$\log x_{50}$ = oxygen pressure at half saturation; p_{HbO_2} and p_{Hb} , osmotic pressures of oxy and reduced Hb. The formula has one merit, there are no undetermined constants, but the agreement with the data is not close. n is too large. The probable cause of this is that Hb and HbO₂ are not strictly homogeneous and separable substances. A qualitative explanation is that both Hb and HbO₂ exist on the same molecule, Hb₄.

Adair's hypothesis is that the molecule Hb₄(O₂)₄ is built up and broken down in stages. Unfortunately this hypothesis does not lead to a formula for the oxygen dissociation curve. An exploration of various possibilities was made and the results reported below, but no quantitative conclusions were arrived at.

The general formula for the building up of the 4 oxygen molecules was deduced for the ideal solution where all components obey the mass law.

$$\frac{0.25K_1x + 0.5K_2x^2 + 0.75K_3x^3 + K_4x^4}{1 + K_1x + K_2x^2 + K_3x^3 + K_4x^4} \quad (4)$$

If the K terms are deduced in the simplest possible manner from the kinetic theory, the following results are obtained.

$$K = 1, K_2 = 3/8, K_3 = 1/16, K_4 = 1/256$$

Substituted in formula (4) these give formula (5).

$$y = \frac{0.25K_1x}{1 + 0.25K_1x} \quad (5)$$

It is known that this formula is not correct, so it is necessary to consider the possible disturbing influences. No theory can be developed, but a suggestion may be considered, in the hope that it may be of assistance in visualizing the points at issue.

The 4 iron atoms may be close together and the system with four oxygens may be very much more stable than the intermediates. Formula (6) gives the type of relation which might be expected with this distribution of iron atoms.

$$y = \frac{0.25(Kx) + 0.25(Kx)^2 + 0.25(Kx)^3 + (Kx)^4}{1 + Kx + 0.5(Kx)^2 + 0.333(Kx)^3 + (Kx)^4} \quad (6)$$

This is written below in the abbreviated form

$$y = f(Kx) \quad (7)$$

It is hardly necessary to detail the steps in the derivation of equation (6) because so many relations are possible that it would be a matter of chance if the details were correct. The formula proves the proposition that n by the oxygen curve *may* be smaller than n measured by osmotic pressures. The simplified formula (7) is useful in later stages of the work.

It will be seen that although the oxygen dissociation curve has been attacked by many workers, no solution has been put forward which gives a completely satisfying reason why the curve has a form adapted to the needs of the organism, rather than the form given by the simple laws of physics and chemistry.

Hyperbolic Dissociation Curve in Distilled Water.

The well known rectangular hyperbola in distilled water, executed by Barcroft and Roberts (2), has played a great part in the development of the theory of hemoglobin solutions. It is the foundation stone of the theories of Prof. Hill and Mr. J. B. S. Haldane. It appears to contradict the theory of Adair.

It is quite certain that in the presence of acids hemoglobin

does change its state of aggregation, and the theory that the low n in distilled water is due to the formation of unimolecular hemoglobin is extremely probable. The many arguments in favor of unimolecular hemoglobin make it very necessary to consider the case for the constant molecular weight of Adair's theory.

The data given below show that when corrections are made for the change in acidity on oxygenation, the hyperbolic curve is not obtained. The corrected value of n is about 2, practically the same as in blood. Hence there is no need to assume that unimolecular hemoglobin is formed in the dialysis of human hemoglobin.

The effect found by Barcroft and Roberts is a little larger than can be accounted for by the correction given below, so there

TABLE IV.

Corrections for Distortion by Acid Change on Oxidation.

$x_1 = 3$ mm., $y_1 = 22$ per cent, x_2 observed = 22 mm., $y_2 = 87$ per cent = $\delta y = 0.87 - 0.22 = 0.65$. Therefore $\delta \log x = 0.189$, $\log x_1 = 1.342$. $\log x_2$ corrected = $1.342 - 0.189 = 1.153$. $\log \frac{y_1}{1 - y_1} = -0.550$. $\log \frac{y_2}{1 - y_2} = 0.826$. $\delta \log \frac{y}{1 - y} = 1.376$. $\log x_1 = 0.477$. $\log x_2$ uncorrected - $\log x_1 = 0.865$, $\log x_2$ corrected - $\log x_1 = 0.676$. n uncorrected, $\frac{1.376}{0.865} = 1.59$, n corrected, $\frac{1.376}{0.676} = 2.04$.

may be specific differences in this matter, but the data of Table IV justify the conclusion that the state of aggregation of human hemoglobin is the same in distilled water and in salt solutions.

It will be seen that the n of the curve (Adair, Barcroft, and Bock) when uncorrected is 1.59, a figure about the same as the n of the curve of Barcroft and Roberts. When the correction for the pH change on oxidation is made, n rises to 2.04.

Dissociation Curves at the Isoelectric Point.

The curve at pH near 6.6 is the nearest approach to pure hemoglobin. The value of n is about 2.12, and the value of x at 50 per cent is about 33 mm. This figure is much larger than the previously accepted figure for the half saturation pressure

of pure hemoglobin, about 8 mm., but the old figure was probably obtained on a preparation of sodium hemoglobinate. Adolph and Ferry's result is $x_{50} = 25 \pm 5$ for horse hemoglobin.

Dissociation Curves on the Acid Side of the Isoelectric Point.

The curve marked pH 6.0 on Fig. 1 and the data of "May 14, Table I," are of interest for two reasons. First, the solution was unaffected by CO_2 . Since more acid did not affect it, the curve must be near the position of minimum affinity for oxygen. For this reason it was used as a base line for measuring the extra

TABLE V.
Theoretical and Observed Dissociation Curves.

$$y = \frac{0.25K_1x + 0.5K_2x^2 + 0.75K_3x^3 + K_4x^4}{1 + K_1x + K_2x^2 + K_3x^3 + K_4x^4} \quad (4)$$

Where $K_1 = 1$, $K_2 = 0.5$, $K_3 = 0.3$, and $K_4 = 1$.

x calculated.	y calculated.	$\log x$ calculated.	$\log \frac{y}{1-y}$ calculated.	$\log x$ observed.	$\log \frac{y}{1-y}$ observed.
0.1	2.5	0.000	-1.591	0.74	-1.42
0.2	5.2	0.301	-1.265	1.06	-1.07
0.5	16.3	0.699	-0.710	1.37	-0.61
0.8	33.6	0.903	-0.297	1.42	-0.55
1.0	45.6	0.000	-0.075	1.75	+0.18
1.2	57.0	0.079	+0.122	1.86	+0.44
1.5	69.8	0.176	+0.364	1.94	+0.55
2.0	82.3	0.301	+0.667	1.70	+0.06
2.5	88.8	0.398	+0.900		
3.0	92.2	0.477	+1.073		
4.0	95.6	0.602	+1.336		

$\log x$ calculated + 1.65 = $\log x$ observed.

affinity for oxygen caused by changing the pH. In Table II the affinity is calculated by deducting $\log x$ of the solution measured from $\log x$ of the acid preparation at the same value of y .

Second, the data were used to test formula (6). This was done by two methods. In Fig. 1 the theoretical values of x and y were calculated and the data marked with crosses. In Fig. 2 $\log \frac{y}{1-y}$ and $\log x$ were calculated and the observed data plotted. This figure shows how the formula produces the curious upward

curvature of the logarithmic curve. The data are collected in Table V. The two series differ by a term which is approximately constant. If Hill's theory was correct, the points of Fig. 2 should fall on a straight line, so it is clear that a theory rather more complex than Hill's is required to explain the facts. The number of constants in equation (6) is so large that this agreement has little theoretical significance.

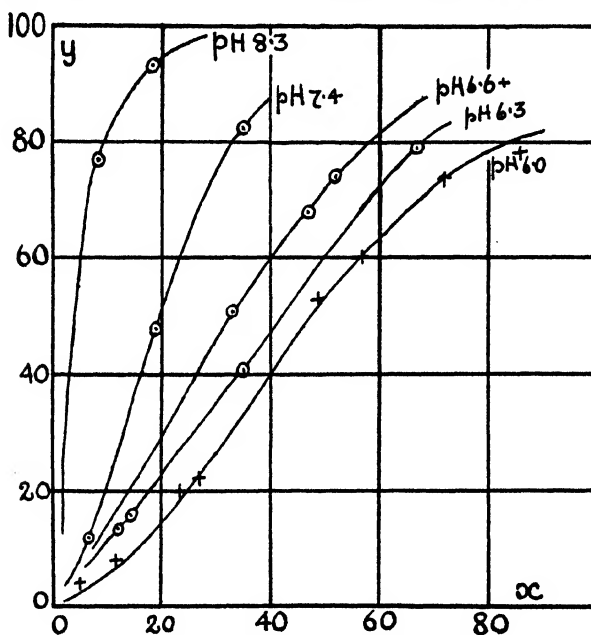


FIG. 1. Oxygen dissociation curves at different H ion concentrations. y = percentage of oxygenation. x = oxygen pressure in mm.

Effect of the OH Ion on the Affinity of Hemoglobin for Oxygen.

The data of Table II show that the pH practically determines the affinity of hemoglobin for oxygen. There is some variation with changes in the NaHCO_3 concentration, but these are small compared with the effects of pH. This conclusion is borne out by Fig. 4, where all the points are plotted. Some variation is present, but the variations due to differences in NaHCO_3 are of minor importance.

Formula (6) can be written in the abbreviated form

$$y = f(Kx)$$

The affinity of hemoglobin for oxygen is proportional to the log of the equilibrium constant, K .

If the K ratios of formula (6) are constant, a very simple method of calculating affinity can be devised, for at any given saturation Kx must be constant, therefore the change in $\log K$ equals minus the change in $\log x$.

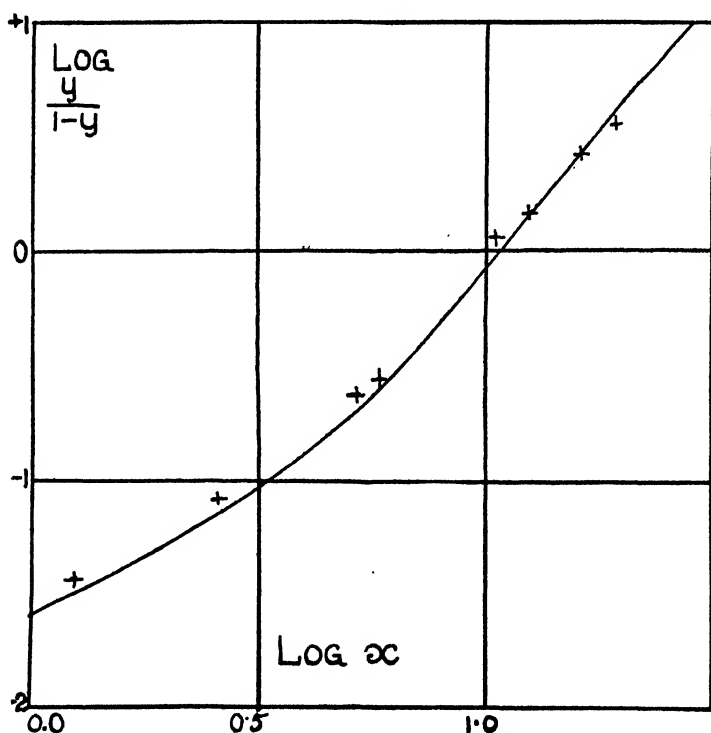


FIG. 2. Test of formula (6). Curve drawn from 6 experimental points from Table IV.

Taking the standard acid curve as a base line, the increase in $\log K$ with pH can be obtained by subtracting $\log x$ of the alkaline solution from $\log x$ of the acid preparation at the same value of y . This gives A , the change in affinity.

$$A = \log x \text{ acid preparation} - \log x \text{ alkaline solution.}$$

A can be expressed in calories by multiplying by $2.303 RT$. The maximum range in A is about 1, corresponding to 1,420 calories. The relation of A and pH is shown in Fig. 3.

It will be seen that the affinity A increases steadily with the pH. In the physiological range, pH 7.2 to 7.8, the relation is nearly linear, a result which agrees with Barcroft and Peters' rule for blood.

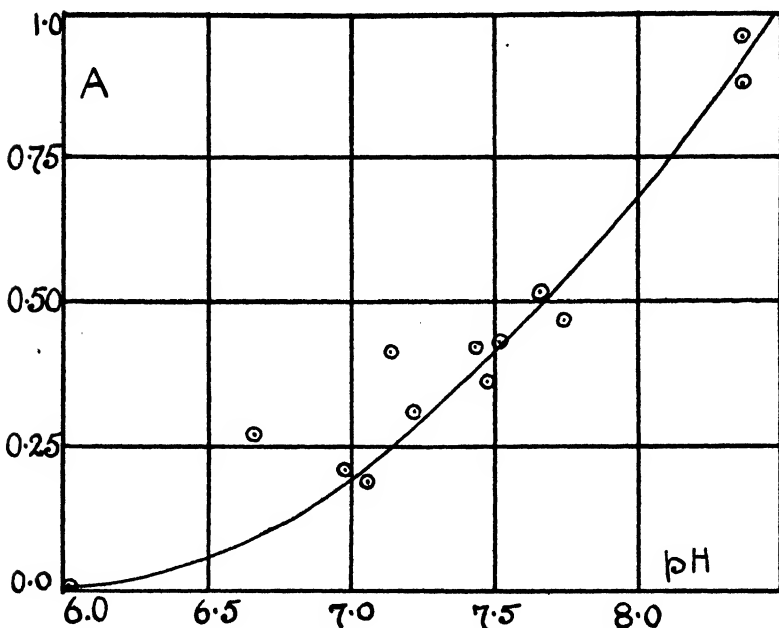


FIG. 3. Effect of pH on affinity for oxygen. Ordinate A (affinity) = $\log K$ (equation (7)) = $1.68 - \log x$ at $y = 50$ per cent.

Taking the wide range, it will be seen that the relation between A and pH is a curve and not a straight line.

Theoretical Calculation of Base Bound on Oxidation from the Measured Relation of Oxygen Tension and pH.

In a previous paper, the base bound on oxidation was determined from the difference of the total base bound to oxyhemo-

globin and the total base bound to reduced hemoglobin at the same pH.

Assuming the theory of non-stoichiometric reactions (Adair (20)), it should be possible to calculate this quantity from measurements of $[\text{OH}]$ ions and O_2 pressures, without any direct estimates of base bound. Let Z = mols of base bound per mol of Hb oxidized. Then applying equation (3)

$$Z = \frac{-d \log [\text{O}_2]}{d \log [\text{OH}]}$$

Therefore,

$$Z = \frac{dA}{dpH}$$

TABLE VI.

pH	Z calculated.	ΔB observed.
6.0	0.10	
6.6	0.20	0.08
7.0	0.40	0.41
7.2	0.43	
7.4	0.46	0.51
7.6	0.51	0.56
7.8	0.57	0.55

Hence Z can be calculated by drawing tangents to the curve of Fig. 3. These values are tabulated in Table VI with values of ΔB —the observed values of base bound—from Table VI of Paper V.

This table is a most striking confirmation of the theory of non-stoichiometric reactions. The deviations observed are well within the limits of experimental error.

Heat of Reaction of Oxygen and Hemoglobin.

Brown and Hill's (19) work on the heat of reaction of oxygen and hemoglobin forms a strong argument in favor of Hill's theory.

In presenting an alternative theory it is necessary to prove that the heat of reaction data are consistent with it.

Hill shows that if the oxygen concentration is maintained constant, and y is the saturation at temperature T_1 and y_2 at T_2 , then

$$\frac{y}{1-y} = \frac{K_1 x^n y_2}{1-y_2} = k_2 x^n$$

$$\log K_1 - \log K_2 = \frac{\log y_1}{1-y_1} - \frac{\log y_2}{1-y_2}$$

This result can be applied to calculate q from the formula of van't Hoff.

$$\frac{d \log K}{dT} = - \frac{q}{RT^2}$$

The calculated q is equal to nQ , where Q is the observed heat. It is impossible to use a method exactly like Prof. Hill's with formula (6), but a very simple method can be devised based on formula (7).

TABLE VII.

Temperature. °C.	Saturation. y	Pressure. x	Pressure at 0°.	
			x_0	$\log x_0$
21.5	14.7	4.6	4.3	0.63
	65.1	13.6	12.6	1.10
	96.4	35.3	32.7	1.51
37.5	16.0	14.0	12.3	1.09
	41.2	34.9	30.7	1.49
	61.5	62.6	55.0	1.74
48.0	78.9	67.3	58.2	1.76
	17.6	35.9	30.5	1.48
	28.7	51.5	43.8	1.64
	31.4	53.6	44.6	1.65

If the percentage saturation is maintained constant, Kx must be constant, therefore $\log x + \log K$ must be constant. Therefore $d \log x = -d \log K$.

Table VII gives oxygen dissociation curves of dialyzed hemoglobin, prepared from the blood of Dr. H. Field. This experiment was suggested by Prof. Henderson. These data are shown in Fig. 4. On account of the slight uncertainty of the middle curve, the variation of Q with temperature is not estimated, but the average Q value can be determined.

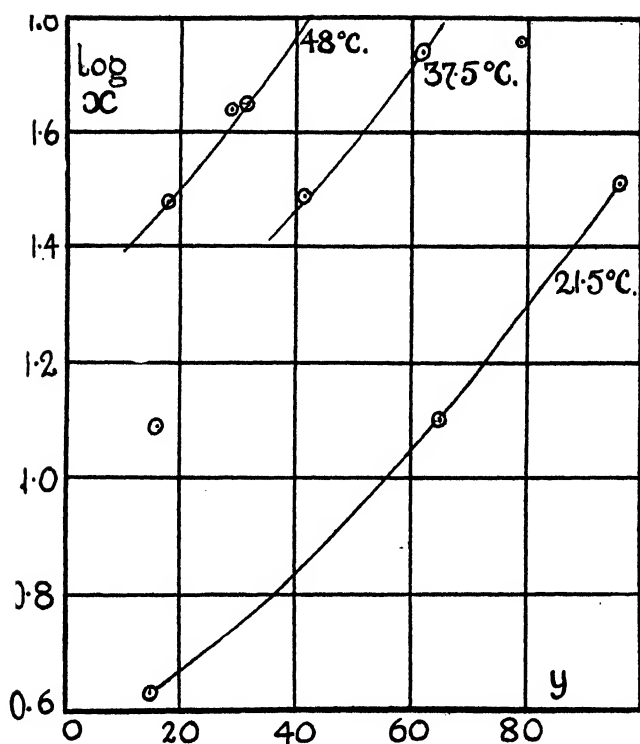


FIG. 4. Influence of temperature on oxygen dissociation. Ordinate = log of oxygen pressure; abscissa, y , percentage of saturation.

Calculation of Q .

$$T_2 - T_1 = (48^\circ - 21.5^\circ) = 26.5$$

$$\log_e x_2 - \log_e x_1 = 3.46 - 1.525 = 1.935 \text{ at } y = 20 \text{ per cent.}$$

$$d \log_e \frac{K}{dT} = - d \log_e \frac{x}{dT} = - 0.0731 \text{ (mean value).}$$

$$- Q = d \log_e \frac{K}{dT} (RT^2)$$

$$R = 2$$

$$T = 34.7^\circ \text{C.} = 307.7^\circ \text{ absolute. } T^2 = 94,500$$

$$Q = 2 \times 0.0731 \times 94,500 = 13,600 \text{ calories.}$$

This calculated value of Q may be compared with the measured value of Q of different observers.

Q	Observer.	Remarks.
13,000-19,000	du Bois-Reymond (21).	
11,600	Berthelot (22).	
10,000	Adolph and Henderson (23).	
11,500	Brown and Hill (19).	34°C. Boric acid blood.
15,000	" " " (19).	34°C. CO ₂ -free blood.
27,000	Barcroft and Hill (16).	34°C. No salt, pH 8 (?).

It will be seen that increase of pH increases Q as it increases affinity. Removal of salts also increases Q as it increases the affinity A .

Our experiments on a purified hemoglobin would be intermediate in pH between the acid blood of Brown and Hill and the CO₂-free blood, and it will be seen that Q calculated from the K of formula (7) lies between 11,500 and 15,000.

TABLE VIII.

Preparation No.	Letter.	O ₂ capacity.	HbO ₂	α at 50 per cent saturation.
Man 1.....	A	22.5	0.010	12.3
" 2.....	B	19.6	0.0088	13.3
" 2.....	B	9.8	0.0044	12.2
Horse 1.....	C	15.0	0.0067	7.0
" 1.....	C	7.5	0.0033	6.5
" 2.....	D	21.9	0.0098	11.8
	D	7.3	0.0033	11.5

In order to calculate Q from the effects of temperature on the dissociation curve, it is necessary to determine n if Hill's method is used. The method based on Adair's formula gives Q directly; therefore, the advantage of simplicity lies with the latter.

Effect of Salts on Affinity at Constant pH.

The data of Table III were graphed and the half saturation pressures were estimated. O₂ capacity = oxygen capacity. HbO₂ = mols of O₂ per liter of solution. The solvent was K(0.1 M), Na (M/7.5), Cl(0.1 M), PO₄(M/15), pH 8.08, corrected for the Donnan equilibrium pH = 8.03.

SUMMARY AND CONCLUSIONS.

Tables of data on oxygen equilibrium curves of pure human hemoglobin are given, and a brief analysis of various theories is made.

Adair's measurements of osmotic pressure can be reconciled with the oxygen data by the theory that the complex molecule $\text{Hb}_4 (\text{O}_2)_4$, or $\text{Hgb} (\text{O}_2)_4$, is built up and broken down to Hgb and 4O_2 in stages.

Methods of correction for the acid change on oxidation are worked out, proving that the curve in distilled water is practically the same shape as in blood, rather than the well known hyperbola of the law of mass action.

The linear relation between pH and affinity for oxygen found by Barcroft and Peters for blood was found to apply to homogeneous hemoglobin solutions only over a narrow range. It was shown that the base bound on oxidation ΔB could be calculated from this curve, applying the equation given below (Adair (20)).

$$\Delta B = -\frac{\delta \log [\text{O}_2]}{\delta \text{pH}}$$

Data are given on the effects of hemoglobin and salt concentration and also temperature on the affinity for oxygen. The heat of reaction of pure human hemoglobin is about 13,600 calories per mol of oxygen.

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TECHNIQUE IN THE USE OF THE RAT FOR VITAMIN B STUDIES.

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In a paper published in 1923, Steenbock, Sell, and Nelson (1) criticized the technique which several investigators have employed, in which the rat was used as a test organism for determining the vitamin B content of different foodstuffs or of extracts. They point out that starch of high grade commercial purity is essentially free from vitamin B, as are also fats and agar-agar, and that so far as contamination of food constituents is concerned, the protein alone is to be suspected. They describe McCollum's method for the purification of casein (2). They also found casein prepared in this way to be free from vitamin B.

The main point which Steenbock and his coworkers lay stress upon as a source of error in the work of others, is that the eating of excreta supplies a constant source of vitamin B, and so leads to erroneous conclusions when the outcome of feeding tests is interpreted as turning on the content of vitamin B in some preparation which is added to the food mixture for the specific purpose of providing this substance. Muckenfuss (3) pointed out that vitamin B is probably excreted in the urine.

The importance of maintaining the highest possible standards of experimental technique is so fundamental that we were concerned when we read the paper mentioned, in which it was stated that satisfactory results could be obtained in vitamin B studies only when rats are kept on screens, such that the excreta fall through and are no longer available to the animals. We examined our records of such studies in order to find whether we had fallen into error, since in our experiments the rats were kept in

cages, the bottoms of which were covered by pans in which shavings were kept to prevent the animals from coming into contact with metal.

Steenbock, Sell, and Nelson presented a chart¹ showing the records of four rats fed a diet consisting of purified foodstuffs supplemented with 8 per cent of butter fat and 3 per cent of wheat germ as the sole sources of vitamins A and B respectively. The animals were kept in cages provided with false screen bottoms. After 2½ months, during which the animals grew poorly, the false screen bottom was removed without cleaning the cage and the rats were given access to their excreta. Growth was promptly resumed. The authors make no statement as to whether the cage was cleaned after the experiment was begun; that is, whether feces derived from the diet of the rats before being placed in the experimental cages were allowed to collect in the cage, and remain until the screen was removed, or whether the feces eaten were derived solely from the diet containing no other source of the vitamin B than the 3 per cent of wheat germ.

Insight into their method of conducting such experiments, however, may be gained from their statement that: "As they were fed according to the group system they were confined in a rather large cage measuring 2 feet square by 20 inches high. With such a large floor space the cages do not become filthy rapidly and the bedding remains dry. For this reason they were not cleaned oftener than at intervals of several weeks and excreta for consumption were generally available."

In order to check up on our technique we kept a series of animals on a diet deficient in vitamin B, and another series on a diet deficient in vitamin A, each group divided into two lots, one of which was kept on shavings as in our usual practice, and the other on a screen with mesh large enough to permit the dropping through of excreta (2 meshes to the inch). The results were estimated on the basis of the amount of growth observed, and on the number of fecal units counted daily in each of the cages, with and without screens. The total dry weight of the feces from the different groups was recorded at the end of the experiment.

One group was fed the diet which we have long used for prepar-

¹ Steenbock, Sell, and Nelson (1), p. 402.

ing animals for testing preparations for the presence of vitamin B. This diet consists of casein 18.0, salts (185) 3.7, agar-agar 2.0, dextrin 71.3, and butter fat 5.0 per cent respectively. Seven rats on a screen were confined to this diet for 28 days. Another seven rats comparable in every way were kept on shavings. The animals kept on the screen deposited 77 gm. of dry matter as feces during the 28 days. From the seven rats on the shavings, with access to their excreta, we collected during the same interval exactly 100 gm. of dry feces.

The counts for the first 6 days were greater for the rats kept on the screen. Thereafter, more feces were secured from the rats on the shavings than from the rats on the screen. The count of the fecal units was not considered of great significance, since a sufficient number were broken and the sizes of different units varied sufficiently to convince us that the dry weight of feces over the entire period was a much more reliable criterion as to the amount eliminated.

A similar experiment was conducted with a diet deficient in vitamin A. Five rats were kept on a screen and five on shavings. The period of observation covered 74 days. During this time the dry weight of the feces from those on the screen was 265 gm., and from those on the shavings 305 gm.

The animals on the screen on the vitamin B-deficient diet were in much poorer condition at the end of 28 days than were those kept on shavings. It seems hardly justifiable to attribute this to deprivation of excreta, since the feces were removed daily from the shavings cage, and the fecal output was certainly greater in the latter than in the former.

Although there was not the marked difference between the two groups of animals seen in the case of vitamin B starvation, the rats on the vitamin A-deficient diet failed on the shavings earlier than did those on the screen.

We observed that the screen on which the rats were kept had to be washed daily to prevent it from becoming foul. Rats kept on screens are not so comfortable as rats kept on shavings, since they are constantly in contact with a good conductor of heat, and their abdomens are exposed to draughts of air.

We fed two groups of rats a satisfactory diet for 8 months and kept one group on shavings and the other group on a screen. When

fed a satisfactory diet rats apparently tolerate the discomfort, since the growth curves of the two groups were comparable. We believe that discomfort is one factor favoring early failure in rats which are in an unstable nutritive condition such as might result from restricting them to the minimal amount of any vitamin.

We have fed many hundreds of little rats the following diet: casein 18.0, salts (185) 3.7, agar-agar 2.0, dextrin 71.3, and butter fat 5.0 per cent to prepare them for testing the presence or absence of vitamin B in preparations. This diet is free from vitamin B. We have kept them in cages, the bottoms of which were covered by a pan $18 \times 22 \times 2$ inches, containing shavings. The animals increase slightly in weight for about 2 to 3 weeks, then begin to decline; in no instance have we seen continued growth at a slow rate. Nearly all animals are ready to be used at the end of 4 weeks. It is very rarely the case that one is not ready by the end of the 5th week. The explanation of our results, and those of Steenbock and his coworkers, are to be accounted for, we believe, in the different system of management of our colony. A group of young rats started on an experiment at any time during the week always have their cage cleaned on the following Monday. The feces which are deposited during the first few days after they have been taken off the stock diet are, therefore, no longer available to them. Since all experimental cages are cleaned once in 2 weeks thereafter, any vitamin content of the feces derived from vitamin-containing food is very small after the first 3 to 6 days, and practically non-existent after the second cleaning of the cage. Steenbock, Sell, and Jones (4), in an extensive series of experiments, have shown conclusively that there is little ability on the part of the rat to store vitamin B. The weights of dry excreta collected in the test experiments which we have made with rats kept on screens and others kept on shavings, appear to indicate that there is little tendency for the animals to practice coprophagy unless feces are available which have a vitamin value.

The view that our system of experimental work does not involve an error of any appreciable magnitude, as suggested by Steenbock and his coworkers, is supported by the observations of Dutcher and Francis (5), who state that: "if rats are placed on screens for two weeks and then the screen removed, allowing the rat to commence feces eating at that point, the feces seem to have lost

their stimulatory properties. This would indicate that the rat does not possess the power to store the vitamin for long periods."

In the experiments of Steenbock upon the results of which he criticizes the method which has been much used in the type of experiment here under discussion, the feces derived from the diet used before the beginning of the experiment were allowed to remain in the cage for long periods, and so could be eaten by the rats after they were beginning to suffer from vitamin deficiency. Their practice of leaving all excreta in a large floor space until the cage became filthy accounts for the disturbing effect of feces-eating on their experimental results.

Although the rat is thoroughly domesticated, there can be no reasonable doubt that in studying the effects of diets of different kinds upon it, the animals should be made as comfortable and contented as possible, since discomfort makes them unquiet and leads to failure to rest well. If proper attention is paid to cleaning the cages there seems no reason to justify keeping the animals upon large mesh screens, as has been recommended.

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THE EFFECT OF ADDITIONS OF FLUORINE TO THE DIET OF THE RAT ON THE QUALITY OF THE TEETH.*

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As a result of special studies in relation to various nutrition problems we have observed the widest possible differences in the quality of the teeth of many of our experimental rats. In some groups where the nutrition was excellent throughout life we have seen teeth which lasted until the animals were advanced in age without showing signs of decay, or disease of the attaching tissues or apical abscesses. They were essentially perfect. In other groups the teeth would be defective and would early show lesions of the kinds mentioned, although the general nutrition of the animals during early life appeared to be satisfactory. The effects of faulty diets on the teeth were always more pronounced in the incisors, which in the rat grow from persistent pulp throughout the life of the animal. It has proven an exceedingly difficult problem to decide in many cases why the teeth of these animals should have been as defective as they were.

Since all investigators who have examined the teeth and bones for fluorine have found considerable amounts of this element, we were led to consider whether perhaps a deficiency of fluorine in the food might lead to the formation of teeth which had poor structure, and consequently possess little power to resist the

* Since this work was reported at the meeting of the National Academy of Sciences at Cambridge, Mass., in November, 1924, the paper of Schulz and Lamb (Schulz, J. A., and Lamb, A. R., *Science*, 1925, lxi, 93) has appeared in which they report the results of feeding sodium fluoride to rats.

agencies which lead to decay. We have in no instance attempted to control the intake of fluorine in any of our experimental animals, so that the amount of fluorine ingested may have varied over a wide range depending upon the nature and amount of natural foods in the diet. Our diets in many cases consisted in considerable measure of purified foodstuffs, so the amount of fluorine ingested in these diets was very small. The possible beneficial effect of adding small amounts of several inorganic elements which are of widespread occurrence but which have generally been regarded as being accidental constituents of the animal body has been suggested by Osborne and Mendel (1), but their experimental evidence gives no definite information concerning a need by the body for fluorine. We were led, in seeking for an explanation for the cause of defective teeth, to study the effect of adding small amounts of fluorine to certain of our experimental diets used with rats.

In making a decision as to the amount of fluorine which should be included in the food mixtures, we were guided by the data relating to the amounts of this element in natural foods, waters, and the body tissues. The following data relating to the distribution of fluorine will serve to help one to gain an idea as to the importance of the amounts ingested by our rats, in terms of what wild or domesticated animals are likely to secure.

Tammann (2), using a method by means of which 0.1 mg. of fluorine could be detected, found the element to be present in the ash of 114 gm. of egg albumin. He found 11.7 parts per million of fluorine in egg yolk, 7.4 parts per million in calf brains, and detected the element in blood and milk. His results were expressed in terms of fresh material.

Zdarek (3) analyzed the tissues of two men, killed in accidents, and found the following amounts of fluorine: in the heart, 4.6 parts per million; kidney, 15.4 parts per million; brain, 2.7 parts per million; lung, 2.2 and 7.0 parts per million; spleen, 8.2 and 23.5 parts per million; liver, 6.8 and 8.0 parts per million. These results are calculated to a dry basis.

Gautier and Clausmann (4) published analyses of 63 samples of vegetable tissues, mostly of food plants. The extreme values found were 5.9 parts per million and 1,380 parts per million, the average for all samples being 265 parts per million. The fluorine content was highest in leaves of plants, but was not characteristic of any particular order. The skins of apples and bananas were found to contain over ten times as much fluorine as the pulp.

Carles (5) analyzed 93 samples of mineral waters, including sea water,

and found fluorine in all but five. The values varied from 4.5 to 79.0 parts per million. Vichy contained 6.0 parts per million. Fluorine is reported to be sometimes added to wines. Carles (6) also found 120 parts per million of fluorine in oyster shells. This is about ten times the content of sea water in this element. He found fluorine in the shells of all the molluscs examined.

Carnot (7) found fluorine in fresh human, ox, manati, and elephant bones in amounts ranging from 1,000 to 3,000 parts per million. In fossil bones he found from 4,000 to 19,000 parts per million. In the teeth of elephants and mastodons of the Pliocene and Miocene ages respectively he found 25,000 to 30,000 parts per million. All the fossil bones contained much more fluorine than modern bones.

Wrampelmeyer (8) found 13,600 parts per million in the sound teeth of an adult, and 6,500 parts per million in the sound teeth of a child as compared with 11,400 parts per million in carious adult teeth, and 15,500 parts per million in the diseased teeth of a child.

Harms (9) asserts that the results of Carnot, Wilson, and Gabriel are too high. He described a method by means of which 0.43 mg. of fluorine could be determined and reports the following values for bones: calf, 50; ox, 50; swine, 180; rabbit, 220 parts per million respectively. In teeth he found the following amounts: calf, 50; swine, 180; dog, 90; man, 60 parts per million respectively.

Jodlbauer (10) criticized the analytical technique of Harms and asserts that his results are too low. He found 1,500 and 1,800 parts per million of fluorine in the bones of a child. In a later paper he confirms the results of Carnot and Wilson. He found bones to contain 500 to 3,200 parts per million of fluorine. The bones of new-born animals contained 1,500 to 1,800 parts per million of this element. Human incisors contained 2,600 to 3,200 parts per million. Molars contained 3,300 to 3,500 parts per million. Tooth germs in dogs contained 4,800 parts per million.

Gautier and Clausmann (11) found the fluorine content of human bones to vary from 150 to 560 parts per million. Animal bones contained similar amounts, but the content was greater in old than in young individuals. The dentine of a dog contained 615 parts per million.

Sonntag (12) examined the bones and teeth of normal dogs and of dogs fed sodium fluoride. He found not over 300 parts per million of fluorine in the bones and teeth of normally fed animals. In animals fed sodium fluoride he found 17,300 parts per million in dried bones and 12,900 parts per million in dried teeth.

Gautier (13) found that in artificial media of known fluorine content the addition of fluorine in most cases favored the growth, flowering, and seed production of plants, especially of *Sinapis*. Its influence was doubtful in rye, wheat, or oats, and in rare cases it was found to be harmful.

Brandl and Tappeiner (14) fed a dog weighing 12.75 kilos varying amounts (0.1 to 1.0 gm.) of sodium fluoride during 647 days. During this time 402.9 gm. of this salt were fed. He found 330.5 gm. in the urine and 72.6 gm. in the feces. No special effects were visible in the dog except a

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stiffness of the back. The dog was anesthetized and the principal tissues were analyzed. He found the following amounts in different tissues.

	NaF in 100 parts of dry substance.	Weight of fresh organ.	NaF content.
	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>
Blood	0.12	750	0.14
Muscle.....	0.13	5,710	1.84
Liver.....	0.59	360	0.51
Skin.....	0.33	1,430	1.98
Skeleton.....	5.19	2,039	59.94
Teeth.....	1.00	25	0.23
Total.....			64.64

Pitotti (15) stated that rabbits and guinea pigs endure neutral solutions of sodium fluoride in "considerable amounts" given by mouth for considerable periods. They became accustomed to it so that a partial immunity was established in that they tolerated doses which would have been toxic to animals unaccustomed to the substance. The kidneys are said to have remained sound. In acute poisoning with sodium fluoride there was degeneration of the epithelium of the kidneys in the region of the convoluted tubules and the loops of Henle. The liver suffered from fatty degeneration and cloudy swelling. The nervous system was greatly affected clinically, but there was no histological alteration. The general nutrition became poor and the number of red blood corpuscles was decreased.

Blaizot (16) found that rabbits tolerated undamaged the injection of a 2 per cent solution of sodium fluoride intravenously up to doses of 0.050 gm. per kilo. Doses exceeding 0.08 gm. per kilo caused symptoms of poisoning. After a 0.1 gm. dose there resulted dyspnea, salivation, polyuria, thirst, diarrhea, and fever. 10 to 15 minutes after the injection coma developed which resulted in death.

Sollmann, Schettler, and Wetzel (17) have recently studied the tolerance of albino rats for sodium fluoride. They fed daily doses of 15 to 150 mg. per kilo of body weight. These amounts resulted in progressive impairment of growth and lessened food consumption. The damage was proportional to the dose, and tended to outlast the administration, since the growth of the animals poisoned with sodium fluoride remained permanently below that of normal animals.

EXPERIMENTAL PROCEDURE.

It is evident from these studies that fluorine is found in nearly all food materials and accumulates in the body tissues. The

high content of fluorine in the teeth suggests that it is one of the structural elements of the enamel and perhaps of dentine. We have carried out experiments to see whether, using certain diets with which we had had much experience, the addition of amounts of fluorine comparable to what might be expected to occur in natural foods would favorably influence the teeth in rats. We tried the inclusion of 226 parts per million of this element in the form of sodium fluoride. We report at this time only the results of feeding a diet which produces good teeth, and of feeding the same diet with fluorine addition. The results showed, contrary to our expectations, that the ingestion of fluorine, in amounts but little above those which have been reported to occur in natural foods, markedly disturbs the structure of the teeth.

The diet of Lot 3619 consisted of: wheat, 67.5; casein, 15.0; whole milk powder, 10.0; NaCl, 1.0, CaCO₃, 1.5; and butter fat, 5.0 per cent respectively. The diet of Lot 3623, containing fluorine, had the same composition, except that sodium fluoride was included to the extent of 226 parts per million. For this purpose 0.1 per cent of a mixture of equal parts of dextrin and sodium fluoride replaced an equivalent amount of wheat.

Those rats fed Diet 3619 had excellent teeth. The ages of the animals, when killed, together with other significant data regarding them, are given in the following table.

Lot No.	No. of animals and sex.	Age at death.	On diet.	Added fluorine.	Remarks.
		<i>days</i>	<i>days</i>		
3619	5 ♀	389	359	0	Animals in good condition.
	5 ♀	392	362	0	" " " "
	5 ♀	473	443	0	Coats somewhat rough.
3623	♂	268	238	+	Overgrown incisors.
	♂	107	77	+	" "
	♂	290	260	+	" "
	♀	127	97	+	" "
	♂	310	280	+	" "
	♀	368	338	+	" "
	2♂	231	201	+	" "
	2♀	231	201	+	" "
	2♂	209	179	+	" "
	3♀	191	161	+	" "
	♂	191	161	+	" "

} Second generation animals.

There was no evidence when the young were taken from the mother that the incisors were faulty. In a few instances, mottled areas were seen, but this has also been occasionally seen in animals where no fluorine has been added to the diet. The females were all fertile and reared practically all their young. The males with which they were kept likewise had fluorine in their food. The animals fed the added fluorine were slightly stunted in that they never grew so large as the controls, and they were shorter and more stocky.

It was observed that the rats fed the diet of Lot 3623 developed incisors which were abnormal in color, the orange tint seen on the anterior surface of the incisors of normal rats being nearly absent. These teeth were also observed to grow into abnormal positions, the upper ones tending in nearly every case to grow backwards into a circle, finally penetrating the roof of the mouth because they were not worn down by attrition. The abnormalities of the incisors were evidently due to changes in their structure and hardness, since the lower incisors tended to wear away too easily, and the upper ones occasionally broke off, leaving a blunt end. The teeth were of especial interest to us in connection with the development of a general plan of study of the relation of the diet to dentition (18). Accordingly, we submitted the skulls of certain animals typical of the collection made by us to Dr. R. W. Bunting, of the University of Michigan, for description. His report is as follows:

In a comparison of the skulls of the rats under the fluorine treatment with those on normal diets, it is apparent that certain marked changes in dental and osseous development have been produced by even this slight modification of the normal diet. From their general appearance the bones of the experimental animals do not seem to be as good in quality as in the normal animal. The skulls appear whiter and their surfaces are manifestly more porous and lack that characteristic luster of normal cortical bone. The rami of the mandibles are somewhat thinner and less prominent than those of the controls. In the maxillæ the dental arches of the fluorine rats are fully 1 mm. narrower in the molar region than those of the normal rats (Figs. 1 and 2). In the mandible of one experimental rat there is an osteoporotic affection of the alveolar process about the third molar which caused that tooth to drop out of its socket (Fig. 3).

The most notable characteristic of the fluorine rats is a marked overgrowth of the maxillary incisors, and a corresponding shortening of the mandibular incisors which elongated in a curved fashion to form an arc equivalent to the greater part of the circumference of a circle.

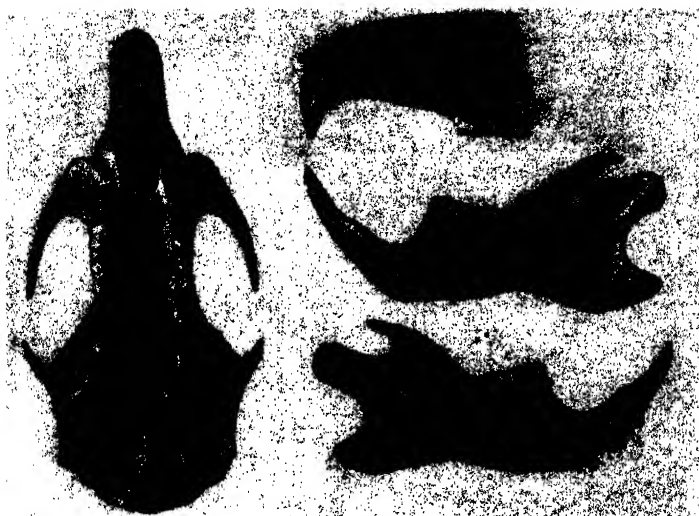


FIG. 1. Normal rat.



FIG. 2. Rat fed sodium fluoride.

The mandibular incisors in one rat have been so severely eroded that the exposed portions of the teeth have been reduced to a level with the gum line. These teeth in the other rat were also excessively worn down, but not to the same extent as in the former. In the latter case the lower incisors were spread widely apart so that they did not come into normal incisal occlusion with their maxillary opponents.

From even a macroscopic examination of the incisor teeth of the experimental animals it is quite apparent that they are inferior in quality to those of the normal animals. They are of a dull, opaque white color and lack the natural polish of well formed tooth substance. In certain areas they are corrugated transversely, suggesting intermittent interferences with development. These departures from normal are more apparent in the



FIG. 3. Mandible of fluoride-fed rat, showing osteoporotic alveolus. \times about $2\frac{1}{2}$.

mandibular incisors, being marked by a wide separation of the teeth and an extreme friability which causes these teeth to be seriously eroded by contact with the maxillary incisors.

At first sight it might be inferred that the great overgrowth of the maxillary incisors has been caused by some stimulative action of the fluorine on the activity of the tooth formative organ. This incisal overgrowth, however, may be accounted for in another way; namely, by the lack of the natural wear which the incisors of the rat under normal conditions exert on each other in occlusion. Donaldson estimates the normal growth of the incisors at 2.5 mm. per week, which amount the adult rat must wear down by incisal attrition to maintain his teeth at the normal length. If, for any reason this incisal reduction by wear does not take place, as when the

upper and lower incisors do not occlude, or when the function of mastication is impaired, the incisors undergo a marked overgrowth quite similar to that present in the fluorine rats. In the experimental animals the mandibular incisors either spread so as to be out of contact with maxillary incisors or, if they did come into occlusion, were promptly fractured so that the maxillary incisors did not have the requisite wear to maintain their normal length. Consequently, these teeth became elongated. (Compare Figs. 4



FIG. 4. Sodium fluoride-fed rats.

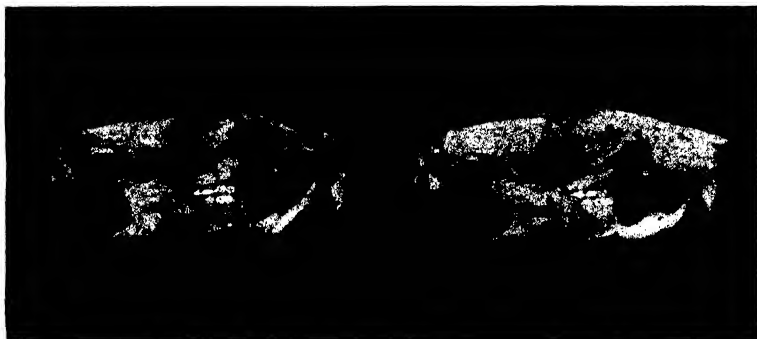


FIG. 5. Normal rats.

and 5.) The manifest inferior quality of these teeth indicates a retrograde disturbance of tooth development rather than a stimulation to overactivity.

The molars of the experimental animals have no marked differences of development from those of the normal. They have prominent cusps and well sealed sulci; they show no evidences of caries; and they differ only in that their enamel surfaces are whiter and have slightly less luster than the normal.

From the results obtained in this experiment it appears that fluorine may play an important part in dental and osseous development and that

the administration of that substance in excess of the normal requirement may have a retrograde rather than a stimulative effect on calcification.

Nutrition investigations have dealt in most instances with the effects of deprivation, complete or partial, of some indispensable nutrient. The cause of defective structure and of low resistance to external agencies, which result in injury to the teeth, has frequently been attributed to deficiency of one kind or another in the food supply. We have, in the present study, a clear demonstration that overingestion of an element which is regularly found in both food and tissues in small amounts may exert a detrimental effect when the amount ingested is increased to but little more than certain samples of foods are known to contain. This is a positive effect of significance in bone and tooth structure.

These results of feeding small amounts of fluorine are so striking that we have undertaken to make similar studies to determine whether small amounts of lead, arsenic, tin, zinc, copper, aluminum, and other elements which are likely to be ingested in traces or in larger amounts, may adversely affect the structure of the teeth.

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THE ENZYMATIC SYNTHESIS OF PROTEIN. IV.

THE EFFECT OF CONCENTRATION ON PEPTIC SYNTHESIS.

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In the enzymatic hydrolysis and synthesis of proteins *in vitro*, the important factor, the factor upon which the direction and the degree of the reaction are dependent, is not the relative concentration of water, but the concentration of material in solution. This conclusion, pointed out by Moore, the authors have discussed at length in a previous paper (1). As shown there, the molecular concentration of water is always so enormously greater than that of the other components that the small amounts added or removed in the course of either reaction are negligible, and it may, therefore, be considered as remaining constant. The distinguishing feature of the hydrolysis and synthesis of protein is the conversion of 1 molecule of protein into a number of molecules of products. It is this characteristic which is responsible for complete hydrolysis in dilute solutions and for the ease with which synthesis is achieved in concentrated solutions. It follows that the extent of synthesis will increase as the concentration increases, and that as the concentration decreases a point will be reached at which synthesis will fail. The concentration at this point will correspond to the maximum concentration of protein capable of complete hydrolysis.

To locate this crucial concentration, two series of experiments were designed. In both series, the amount of protein synthesized in various concentrations of hydrolytic products was compared, but in one series (a) the enzyme concentration varied with the concentration of products, in the other (b) the enzyme concentration was kept constant. The arrangement of conditions in Series a and the results obtained are shown in Table I.

The mixtures, consisting of various dilutions of a peptic hydrolysate of albumin to which pepsin had been added, were preserved with chloroform and were contained in stoppered Erlenmeyer flasks. They were set away at 33°C. for 48 hours. After 48 hours the solutions were neutralized and diluted to 250 cc., and the total nitrogen estimated in duplicate on 25 cc. of the suspension. The protein was filtered off and the total nitrogen estimation again carried out on 25 cc. of the filtrate. The difference between the two was the amount of protein nitrogen.

In 10 minutes at room temperature heavy precipitates appeared in Dilutions 1 and 2 and a slight precipitate, in 3. The other three dilutions showed no noticeable increase in turbidity. After 24 hours at 33°C. a slight precipitate had appeared in 4, while

TABLE I.

Series a.

Effect of Substrate Concentration on Synthesis with Varying Enzyme Concentrations.

Digest at pH 3.9.	Pepsin.	0.0001 N HCl.	Dilution.	N in 100 cc.	Total N.	N in filtrate.	Protein N.
cc.	gm.	cc.		mg.	mg.	mg.	mg.
20	1.01	0	1	6,500	1,300	1,100	200
20	1.01	20	2	3,270	1,310	1,210	100
20	1.02	40	3	2,230	1,340	1,280	60
20	1.01	80	5	1,310	1,310	1,300	10
20	1.00	130	7.5	870	1,300	1,300	0
20	1.00	180	10.0	650	1,310	1,310	0

the protein precipitates in 1, 2, and 3 had become denser; 5 and 6 were still without protein precipitates after 48 hours; and the amount of protein precipitated in 1, 2, 3, and 4 varied visibly, inversely as the dilution. The pH of 4, 5, and 6 was 3.9 at the end of the experiment.

Up to the fifth dilution the amount of protein is inversely proportional to the dilution. The concentration at which no protein formation occurs, at 33°C., lies between 870 and 1,310 mg. of nitrogen per 100 cc.

The arrangement of conditions and the results obtained with Series *b* are shown in Table II.

In all dilutions except 8 and 10 (Table II) the typical protein precipitates appeared in less than 10 minutes. The flasks were

set away at 37°C. for 48 hours; they were then neutralized, diluted, and analyzed for protein in the usual manner. The pH of 7 and 8 at the end of 48 hours was 4.0.

Protein synthesis again ceased at a concentration approximating that of the previous experiment, *viz.* 1,270 mg. per 100 cc., despite an 8-fold increase in the enzyme concentration. This value corresponds to approximately 8.0 per cent of protein. The concentration of protein in plasma is from 7 to 8 per cent. While it is not possible at present to infer any direct connection between these two facts, the similarity in value seems possibly significant.

The results of the two series of experiments (*a* and *b*), cannot be combined in one set of data. Series *a* was carried out at 33°C.

TABLE II.

Series b.

Effect of Substrate Concentration on Synthesis, with Enzyme Concentration Constant.

Digest at pH 3.9.	Pepsin.	0.0001 N HCl.	Dilution.	N in 100 cc.	Total N.	N in filtrate.	Protein N.
cc.	gm.	cc		mg.	mg.	mg.	mg.
10	0.4	1	1	8,050	890	680	210
10	0.8	12	2	4,280	940	800	140
10	1.2	23	3	2,990	990	910	80
10	1.6	34	4	2,360	1,040	990	50
10	2.0	45	5	2,030	1,120	1,070	50
10	2.4	56	6	1,690	1,170	1,130	40
10	3.2	78	8	1,430	1,260	1,250	10
10	4.0	100	10	1,270	1,400	1,400	0

or 4° lower than Series *b* and with a brand of pepsin which gram for gram was less active than that used in Series *b*. The same general conclusions may, however, be drawn quite definitely from both; a straight line relationship between the amount of protein synthesized and the concentration of material in solution (Fig. 1), a falling off from the straight line relationship at high concentrations, and approximately the same minimum concentration for protein formation.

The falling off from the straight line relationship with the higher concentration of material is definite in both, and the same phenomenon to a more marked degree, appeared in experiments designed to effect 100 per cent synthesis.

A mixture of enzyme and digest under optimal conditions (*i.e.*, pH 4.0; 4.0 per cent pepsin and concentrated hydrolysate) was set away in a vacuum desiccator at 38°C. A sufficiently low pressure was maintained to cause the digest to froth into large bubbles. A very great concentration of products and a great diminution in

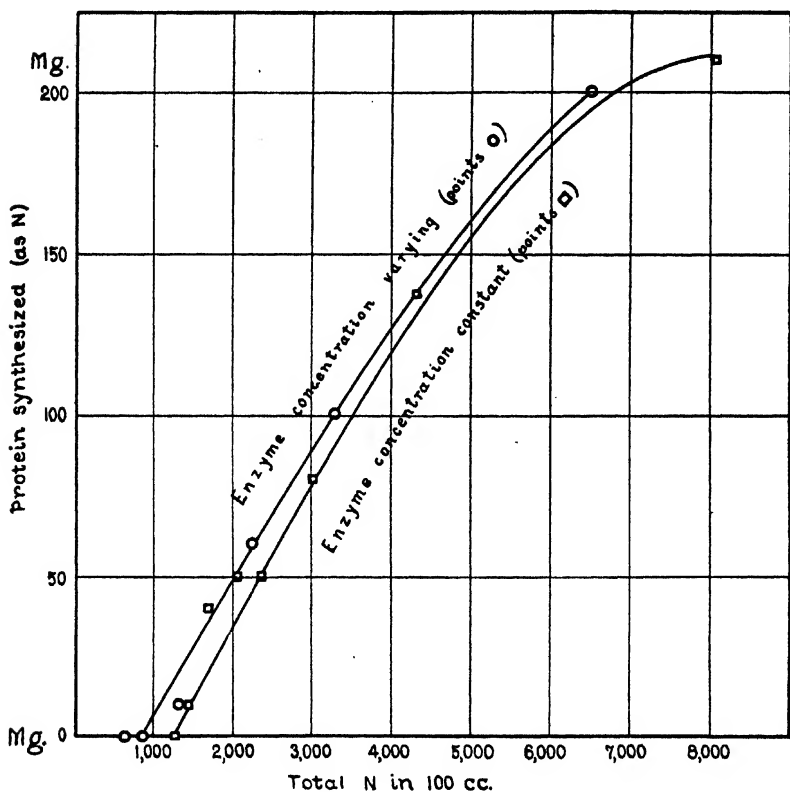


FIG. 1. Relation between amount of protein synthesized and the concentration of material in solution.

the water content was thus achieved, and the mixture was practically dry so that a greatly increased yield may justifiably have been expected. To our surprise, analysis after 48 hours showed only traces of protein to have been formed. In very great concentrations of material not only had there been a relative falling off in the amount of protein, but also a large absolute decrease.

Persisting still in the endeavour to obtain a large yield, we dissolved 0.5 gm. of pepsin (Merck) in 10 cc. of 0.01 N HCl; 10 cc. of concentrated peptic digest at pH 4.0 were added, with chloroform, and the mixture was set away at 37°C. for 3 hours. By that time the digest had become a solid jelly, with the whitish yellow colour indicative of the formation of large amounts of protein. The flask was now uncorked and set in a sulfuric acid desiccator under a slightly diminished pressure, at 38°C. 21 hours later, the vacuum was greatly increased to a pressure of 50 mm. of mercury and the desiccator replaced at 37°C. By diminishing the pressure thus gradually, it was hoped that the evaporation of water from the digest would be slow enough to allow the protein formation to proceed beyond the limits yet attained, before the inhibiting effect of a too low concentration of water could overcome the synthesizing tendency of the increased concentration of products.

At the end of 48 hours the flask was removed and analyzed for protein and 30 per cent was found. The amount of protein produced by 0.5 gm. of pepsin, at 38°C. in 36 hours, without additional concentration, was found to be 31 per cent. Disregarding the difference of 1 per cent, it may be concluded that further concentration of the solution, even to a relatively slight degree, was without any augmenting effect on the amount of protein formed.

A related phenomenon was encountered by Armstrong and Gosney (2) in their experiments on the enzymatic synthesis of fats. They attributed the inhibitory influence of too low concentrations of water to the resulting diminution or removal of the surfaces in the heterogeneous systems of oily substances in water with which they were dealing. In their own words: "Apparently, as pointed out by us previously, the intervention of a film of water is necessary at the interface of the system, where interaction takes place; if this be removed, action comes to an end." This explanation will not suffice here, because all the reacting substances, except the precipitated protein, are in solution.

Another more serious problem arose from the practical coincidence of the minimal concentrations of digest for protein formation at the identical point, with enzyme concentrations varying as widely as 4 and 0.5 per cent. If the minimal concentration of products necessary for synthesis at 38°C. is at approximately 8 per cent, in view of the insolubility of protein it would be expected

that synthesis would proceed at all higher initial concentrations until the concentration had fallen to the critical value of 8 per cent.

Table III shows that the facts are otherwise.

None of the factors which are usually considered to account for the cessation of an enzyme action, appear to be functioning here. All the available substrate was not utilized, or the same amount of protein would have been formed in each; the enzyme concentration was maintained constant in Series *b*, so that variation in the amount of enzyme, which has been previously shown (3) to affect the equilibrium, cannot be the cause; the possibility of auto-destruction of the enzyme has been ruled out experimentally, and previous work had shown that the above figures represent equilib-

TABLE III.

Amount of protein formed in 100 cc.	Concentrated substrate in 100 cc. before protein formation.	Concentrated substrate in 100 cc. after protein formation.
gm.	gm.	gm.
11.9	50.3	38.4
3.9	26.7	22.8
1.5	18.7	17.2
0.7	14.7	14.0
0.5	12.7	12.2
0.4	10.6	10.2
0.1	8.9	8.8
0.0	7.9	7.9

rium amounts and not velocities (3). The speed of protein precipitation was visibly greater in the more concentrated solutions, where the discrepancy between the critical 8 per cent and the final concentration is greatest.

Two possible explanations remain. Either, in the synthesis of protein, enzyme is removed, or the figures represent equilibrium amounts in the specific mass law sense of the term equilibrium. If the latter be true, initial addition of protein, even though, as in this case, insoluble, should inhibit the subsequent synthesis of protein from the digest; and the extent of the inhibition should be proportional to the amount added.

The following experiment was devised in an attempt to test the first possibility. 0.5 gm. of pepsin was dissolved in 0.0001 N

HCl; 7.5 cc. of digest at pH 4.0 and 0.5 cc. of chloroform were added; the flask tightly stoppered, and set away at 37°C. 50 hours later an additional 7.5 cc. of digest were pipetted in, thoroughly mixed with the heavy gelatinous paste of digest and protein already present, tightly stoppered, and set away again at 37°C. This was Flask *A*. Another flask, *B*, containing 0.5 gm. of pepsin and 15 cc. of digest, was incubated for the same total length of time as was *A*. During the period of incubation the mixtures were thoroughly stirred twice in each 24 hours.

After 5 days Flasks *A* and *B* were removed from the incubator and the contents analyzed for protein.

Assuming that enzyme is removed in the course of synthesis; it follows that the total amount of protein formed in *A* will be less than in *B*, because the enzyme, which has promoted the formation of protein in the first portion of digest in *A*, will have been in large part removed. The percentage of synthesis in the second portion will consequently have been low, and presumably the sum of the synthesis in the two portions should be less than if the whole of the enzyme had been allowed to act upon both portions of the digest at once as in *B*. It is probable, of course, that the percentage of protein formed in the first portion of *A* will be slightly greater than would be formed in the whole digest of *B*, on account of the greater concentration of enzyme, but it would also probably not be great enough to compensate for the large falling off in the second portion.

If, on the other hand, there is no difference between *A* and *B*, then we have, to say the least, no evidence that enzyme has been consumed. On analyses, the latter result was obtained.

The amounts synthesized in *A* and *B* were 410 and 402 mg. of protein nitrogen respectively, or 39.3 and 38.3 per cent of the digest N. Practically the same amount of protein was synthesized in each flask and we have no evidence of enzyme removal in the progress of synthesis.

It is to be admitted that this experiment is not conclusive, but it was impossible to devise one less unsatisfactory, and fortunately the second possibility, *viz.* that addition of protein will inhibit synthesis, proved to be readily demonstrable, and it is therefore unnecessary to explain the results by the removal of enzyme.

In a tube, *C*, a suspension of boiled and thoroughly washed

synthesized protein, containing 146 mg. of nitrogen, was centrifuged. The supernatant fluid was carefully poured off, leaving the solid heavy precipitate in the tube. 0.5 gm. of pepsin and 3.5 cc. of 0.0001 *N* HCl were added, the enzyme was dissolved, and then 12.5 cc. of concentrated peptic digest pipetted in. The mixture was thoroughly stirred, and set away at 37°C. A flask, *D*, containing 0.5 gm. of pepsin, 12.5 cc. of digest, and 2.5 cc. of 0.0001 *N* HCl, was prepared and set away at 37°C. at the same time as was *C*. During the period of incubation the mixtures were thoroughly stirred twice in each 24 hours. After 5 days the flasks were removed from the incubator and their contents analyzed for protein.

If the added protein, though not in solution, is nevertheless a component of the system in equilibrium, then the amount synthesized in *C* will be less than in *D*. The results showed this to be the case. In *C*, where protein equivalent to 146 mg. of N had been added, protein equivalent to only 266 mg. or 29.9 per cent of the digest N was synthesized, while in *D*, where no protein was added, protein equivalent to 302 mg. of N or 35.7 per cent of the digest N was synthesized.

The insoluble added protein exerted an inhibiting influence on the synthesis of protein from the digest. Though not in solution it acted as if it were in solution. The total amounts of nitrogen in suspension, and of protein present at equilibrium, in *A* and *C* were identical. The protein, though precipitated, retains the same significance as a compound in solution in its influence upon the condition of equilibrium.

This unexpected conclusion was submitted to a more rigid verification.

Five flasks were prepared as in the previous experiment, and various amounts of thoroughly washed and boiled freshly synthesized protein were added. Water and digest were added in such amounts that the nitrogen concentrations of the suspensions were the same in all. The contents of the flasks are tabulated in Table IV, and the results in Table V. They were incubated at 37°C. for 4 days and then analyzed for protein.

In the last column of Table V are given the lesser amounts of protein that would have been formed in the absence of added protein as a result merely of the dilution introduced in adding the protein.

The figures in the last two columns remove the possible objection that the dilution is the cause of the lower amounts synthesized in the mixtures to which synthesized protein had been added; and the definite result is obtained that the extent of synthesis is less in those mixtures containing added protein than can be accounted for by dilution.

The results in the third and fourth columns strikingly confirm the conclusion drawn from the previous experiment. In Fig. 2 the

TABLE IV.

Digest.	Pepsin.	H ₂ O	Protein N.
cc.	gm.	cc.	mg.
10	0.4	1.0	0
9.5	0.4	1.5	39
9	0.4	2.0	77
8	0.4	3.0	154
7	0.4	4.0	231

TABLE V.

Total N.	N after filtration.	Protein N.					
		Added.	Synthesized.	Added and synthesized.		Synthesized.	Hypothetical synthesis.
		mg.	mg.	mg.	per cent of total	per cent of digest N	per cent of digest N
794	570		224	224	30.2	30.2	30.2
829	595	39	195	234	30.1	26.4	28.7
836	593	77	166	243	31.0	23.5	27.1
807	530	154	123	277	36.6	20.5	24.6
777	474	231	72	303	41.8	14.6	21.1

amounts of protein synthesized are plotted as ordinates against the protein added as abscissæ. They give a steep, straight line.

One unexplained result, however, does remain among those given in Table V. This is shown in Fig. 2 by the failure of the straight line to cut the horizontal axis at an abscissa equal to the ordinate at which it cuts the vertical axis. It would seem that the addition of synthesized protein to a solution of enzyme and digest does not retard subsequent protein formation as much as the synthesis itself.

Addition of a solution of egg albumin to a solution of digest and pepsin inhibited the synthesis of protein in the same manner as synthesized protein itself. The amount of albumin that could be added without diluting the solution too greatly was, however, small, and in addition the albumin was denatured and precipitated as soon as it was mixed. The result, therefore, carries no additional significance beyond confirming the phenomenon already observed; *i.e.*, the inhibition of a reaction occurring between sub-

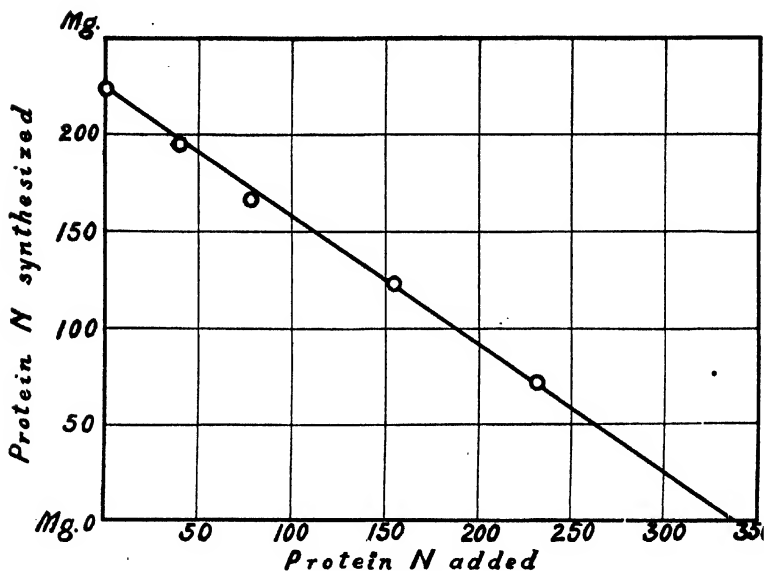


FIG. 2. Relation between the amounts of protein synthesized and the amounts of presynthesized protein added.

stances in solution, by the presence of material, which in the usual sense, is insoluble in that solution.

The above experiments showed that in peptic hydrolysates of egg albumin, in the presence of pepsin, more and more protein is synthesized as the concentration increases above a value corresponding to approximately 8 per cent of protein. Considering the whole nitrogen content as having arisen from the protein present, this conclusion can be restated; that, as the initial concentration of protein increases the degree of hydrolysis decreases. This is

experimental confirmation of a prediction made on theoretical grounds in a previous paper (1). The proof, however, is indirect.

An experiment was carried out to obtain, if possible, direct confirmation. A difficulty was encountered, however, which at present seems insuperable. Solutions of albumin of greater concentration than 8 per cent, in acidities as high as pH 2.0, become jellied and coagulated. On subsequent dilution only part of the coagulum redissolves, and if one attempts to filter the jelly, the bulk of the protein remains on the filter, so that the filtrate is only a dilute solution of protein. This gelatination does not occur with 6 per cent albumin; nor even with solutions as highly concentrated as egg white, *viz.* 14 per cent, when the reaction is neutral or slightly alkaline.

Concentrated solutions of albumin were submitted to peptic hydrolysis at pH 1.6 for a number of days and the degree of hydrolysis noted at intervals of 24 hours. The results obtained have at best only qualitative value; but their tendency is in accord with the predictions made. A 6 per cent solution of albumin was completely hydrolyzed, *i.e.* it contained no demonstrable protein, in 3 days at 37°C. A solution of egg white, 11.2 per cent, freed from membrane, was only 53 per cent hydrolyzed; a 17.4 per cent solution was 44.5 per cent hydrolyzed. In the latter two solutions no change was observed after 48 hours, though the pH was maintained at 1.6. During the first 48 hours, the pH continually rose, as is usual in peptic hydrolysis; and required continual adjustment. After that, for the next 2 days, it remained unchanged.

SUMMARY.

1. In a solution of the products of peptic hydrolysis of albumin, the extent of synthesis with pepsin is in simple inverse proportionality to the dilution.

2. This relationship does not obtain in very concentrated solutions, and in sufficiently high concentrations the amount of synthesis is actually less than in more dilute solutions.

3. With enzyme concentrations varying between 4.0 and 0.05 per cent, synthesis fails at 38°C. in a solution of products which corresponds to approximately 8 per cent of protein.

4. No evidence for the possibility that enzyme disappears in the course of synthesis was obtained.

5. Addition of synthesized protein to a solution of digest and pepsin inhibits the subsequent synthesis to an extent directly proportional to the amount added.

6. A similar inhibition of synthesis also occurs on the addition of native protein.

7. Peptic hydrolysis similarly does not proceed to completion in concentrated protein solutions.

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THE ENZYMATIC SYNTHESIS OF PROTEIN. V.

A NOTE ON THE SYNTHESIZING ACTION OF TRYPSIN.

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In extending our investigation of the enzymatic synthesis of protein to the synthesizing action of commercial trypsin (1) the findings of Henriques and Gjaldbak (2) were reviewed. In their experiments on plastein formation by trypsin, these authors observed a curious simultaneous hydrolysis and synthesis. This observation is confirmed.

It was first necessary to determine the optimum pH for tryptic synthesis.

Solutions were made up as recorded in Table I, using a peptic hydrolysate of albumin as substrate, and adding the amounts of acid, alkali, or water necessary to obtain the required pH and a constant concentration of digest.

The trypsin employed was prepared from "Difco" trypsin. A 10 per cent solution of the commercial product was adjusted to pH 6.5, centrifuged, filtered, and then precipitated with 9 volumes of alcohol. The precipitate was dried, first with alcohol and ether, and finally in a vacuum desiccator. A fine, white product was obtained. The solution was adjusted to pH 6.5, because at that hydrogen ion concentration, as Northrop showed (3), the auto-destruction of trypsin is least rapid. Out of each of the above mixtures two 10 cc. portions were pipetted into 50 cc. Erlenmeyer flasks containing 0.10 gm. of the prepared trypsin, dissolved in 1 cc. of water. The solutions were thoroughly stirred and set away with chloroform in the incubator at 37°C.

Of the sixteen flasks, eight were analyzed for protein at the end of 3 days, and the other eight at the end of 5 days.

At the end of 1 hour, the contents of Flasks 1, 2, 3, and 4 showed precipitates and were almost stiff. Flasks 6 and 7 were immovable transparent jellies. Flask 5 contained some precipitate, but was also somewhat gelatinous.

At the end of 2 days, Flask 7 (pH 9.0) contained a large number of white particles dispersed through the jelly. These, under the microscope, were seen to be composed of crystals resembling those

TABLE I.

No.	Amount of digest.	2.0 N HCl.	2.0 N NaOH.	H ₂ O	pH
	cc.	cc.	cc.	cc.	
1	20	1.5		5.5	5.4
2	20	0.9		6.1	5.8
3	20			7.0	6.5
4	20		0.6	6.4	7.1
5	20		2.0	5.0	7.7
6	20		3.3	3.7	8.3
7	20		5.0	2.0	9.0
8	20		6.4	0.6	9.9

TABLE II.

Effect of C_H on Tryptic Synthesis.

pH	Protein N in per cent of total N.	
	3 days.	5 days.
5.4	12.8	14.0
5.8	13.1	15.4
6.5	11.5	13.2
7.1	9.1	7.9
7.7	7.6	
8.3	8.1	6.6
9.0	6.4	6.2
9.9	5.1	6.7

of tyrosine and cystine, and further evidence that they consisted of these amino acids was obtained from their solubilities, Millon's and sulfur tests. They failed to give the biuret reaction. The particles were present in all the alkaline mixtures, diminishing in number with decrease of alkalinity. The acid solutions contained the typical protein precipitates found in peptic synthesis, and no precipitated amino acids.

At the end of the period of incubation the mixtures were diluted to 100 cc. and 40 cc. of each of the resulting suspensions were pipetted into 10 cc. of 2 per cent trichloroacetic acid. In this concentration of acid, tyrosine and cystine are quite soluble. The mixtures of diluted trichloroacetic acid were allowed to stand for 24 hours in order to allow the precipitated amino acids to redis-

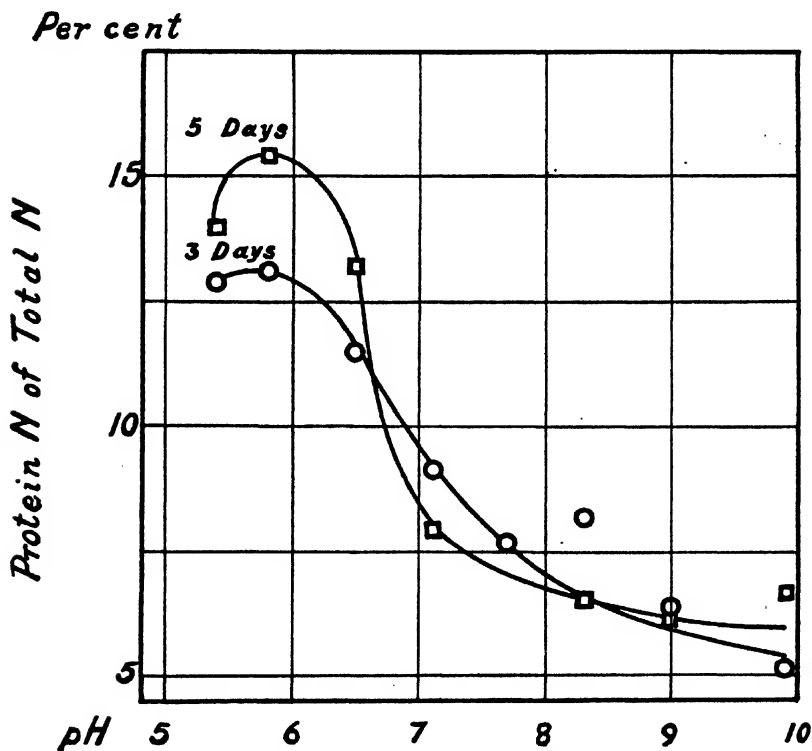


FIG. 1. Relation between C_H and the synthesizing action of trypsin.

solve, and the resolution was facilitated by frequent rubbing and shaking. The precipitated protein was then filtered off and the total nitrogen of the filtrate determined by the macro Kjeldahl method.

The difference between this and the total nitrogen of the original, diluted digest was a measure of the protein synthesized. The results of the analyses of the 3 and 4 day series are stated in Table II and Fig. 1.

The substance synthesized has been called protein because it possesses the general properties of protein. It resembles the protein synthesized by pepsin from a similar peptic hydrolysate of egg albumin. It is very soluble in dilute alkali, less so, but still fairly soluble, in dilute acid. It gives the biuret reaction and is precipitated from its solution in acid by trichloroacetic acid.

The optimum pH for tryptic synthesis, as Fig. 1 shows, is in the neighborhood of 5.7. From the location of the optimum the synthesizing enzyme is clearly not pepsin. In all the alkaline solutions, as Henriques and Gjaldbæk found, a simultaneous hydrolysis ensues. Whether or not both these reactions are promoted by the same enzyme cannot be decided by these experiments. The hydrolysis is strikingly brought out by the greater steepness of the 5 day curve than that of the 3 day curve. Whereas all the flasks on the acid side of pH 7.0 contained larger amounts of protein at the end of 5 days than at the end of 3, on the alkaline side they were found to contain consistently less. The synthesis is a more rapid reaction than the hydrolysis, which is to be expected in solutions of such high concentrations. The hydrolysis following later, and continuing for a longer time, to some extent reverses the synthesis that has occurred.

SUMMARY.

1. The optimum hydrogen ion concentration for tryptic synthesis of protein in a peptic digest of egg albumin is shown to be in the neighborhood of pH 5.7.

2. As had been observed previously by Henriques and Gjaldbæk, in neutral and alkaline reactions, an hydrolysis also occurs simultaneously with the synthesis.

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SOME NITROGENOUS CONSTITUENTS OF THE JUICE OF THE ALFALFA PLANT.

III. ADENINE IN ALFALFA.*

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(Received for publication, February 13, 1925.)

Attention has been recently called, in two papers from this laboratory (1, 2), to the presence in the juice of the alfalfa plant of a base yielding a picrate melting at 298°C. The amounts previously obtained were too small to permit of identification. More of it has now been isolated, and the base identified as adenine.

The most characteristic salt of adenine is the picrate. This is thrown down from a solution of pure adenine or its salt as a voluminous, sulfur-yellow, crystalline precipitate, by the addition of picric acid. The picrate dissolves in approximately 160 parts of boiling water and separates on cooling in long hair-like needles which fill the entire solution. The solubility in water at 15° is of the order of 1 in 8,000. The crystals possess a silky luster and contain 1 molecule of water of crystallization which is slowly lost over sulfuric acid at room temperature and is readily driven off at 105°. The anhydrous picrate retains its silky luster. The solubility in cold absolute alcohol is of the order of 1 in 5,000.

Decomposition Point of Adenine Picrate.—Adenine picrate possesses no true melting point. A pure specimen darkens slightly at 285° and above this temperature decomposition proceeds rather rapidly. Consequently, if the heating is carried out

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The authors wish to express their appreciation of helpful advice from Dr. Thomas B. Osborne.

too slowly the sudden decomposition with evolution of gas may occur many degrees too low or may be missed entirely. When the heating is carried out sufficiently rapidly this sudden gas evolution is very characteristic and takes place at 298° (short thermometer). Our preparations have been heated at a rate of approximately 1° in 3 seconds.

The "melting point" of adenine picrate is given in the literature for the most part at temperatures in the vicinity of 281° . Krüger and Salomon (3) state that it decomposes at 279 – 281° . Bennett (4) gives 280 – 281° and states that adenine picrate when dried "shows no brilliancy." Jackson (5) gives a melting point of 279° and Suzuki, Otake, and Mori (6) give 283° .

On the other hand, Williams and Seidell (7) obtained adenine picrate in *silky needles* melting sharply at 296 – 297° and state that "the decomposition, which begins at about 280° , takes place far from sufficiently sharply to be characteristic." Voegtlin and White (8) prepared adenine picrate from the crystallized sulfate and give melting points from 285° to 289° . Pure adenine picrate is accurately described by Jones and Perkins as "consisting of long, intertwined needles resembling matted hair," but the decomposition point of their preparation is not given (9).

This wide variation in the "melting point" ascribed to adenine picrate can, in our experience, be attributed to two causes. In the first place, adenine picrate, as usually prepared, stubbornly retains a small amount of impurity which lowers the decomposition point and greatly alters the crystalline habit. We have prepared adenine picrate from pig liver, by the conventional methods, which on recrystallization yielded fine needles, grouped in bunches corresponding with the description usually given (10). It decomposed, when rapidly heated, at 295° . This material was converted to the sulfate and a sample of the crystallized sulfate again precipitated as picrate and crystallized. It was then obtained in a voluminous mass of hair-like needles decomposing at 298 – 299° , resembling the picrate from alfalfa in all respects.

In the second place, the observed decomposition point depends to a considerable extent on the rate of heating, as already mentioned. A sample of adenine picrate¹ obtained by the conventional

¹ Prof. L. B. Mendel kindly allowed us to use this specimen from his collection.

methods from embryo chicken livers by Mendel and Leavenworth (11), which was recorded as melting at 282° , was found to decompose at 290° when heated rapidly. This preparation consisted of tiny prisms grouped in balls and was without luster. We therefore emphasize the difference between the crystalline habit, appearance, and decomposition point of adenine picrate as usually described and that of the pure salt.

Preparation of Adenine from Alfalfa.—Adenine was prepared from 23.9 kilos of fresh alfalfa² by the method described in a previous paper (2). This amount of alfalfa contained 4,387 gm. of dry solids and 222 gm. of nitrogen. The "alfalfa filtrate," actually worked up, contained 55 gm. of nitrogen and 870 gm. of organic solids. From this material we obtained 7.583 gm. of adenine picrate, equivalent to 2.891 gm. of free adenine. The yield of adenine is, therefore, 0.012 per cent of the fresh alfalfa plant, 0.066 per cent of the dry plant, and 0.33 per cent of the solids of the alfalfa filtrate. Adenine nitrogen makes up 2.72 per cent of the nitrogen of the alfalfa filtrate. Consequently, while the absolute amount of adenine in alfalfa is small, it forms an important constituent of the juice which bathes the physiologically active tissues.

Adenine in Plants.—The purine fraction of the extracts of plant leaves has not received the attention in the past that the importance of purines in metabolism warrants. Adenine has been found in tea leaves (12), sugar beet juice (13), bamboo shoots (14), and mulberry leaves (15), and is probably quite widely distributed among the green plants. Whether or not the adenine so found is related to the nucleic acid metabolism of the plant is unknown. If it is, we should be justified in expecting to find guanine and cytosine in the plant juice as well. While it is not yet possible to assert that guanine does not occur in alfalfa juice, no indication of its presence has been found. A purine which gives the murexide test has been found in small amounts, but it is

² That the adenine found in alfalfa filtrate was set free by the action of enzymes from some more complex compound during the preparation of the material is, of course, a possibility, but we have no evidence in support of such a view. The juice expressed from the ground plant was promptly heated to 90° with the object of coagulating the protein and limiting enzyme action as much as possible.

582 Nitrogenous Constituents of Alfalfa. III

soluble in ammonia; traces of still other purine-like substances are present. Cytosine has not yet been sought for in alfalfa juice.

EXPERIMENTAL.

The adenine picrate obtained from alfalfa filtrate was decomposed with hot 10 per cent sulfuric acid and picric acid removed. The solution was diluted and sulfuric acid exactly removed. On concentration to small volume adenine separated as a microcrystalline powder. From this preparation the sulfate and chloride were obtained by the addition of acid to a faint acid reaction to Congo red. The sulfate crystallizes in square

TABLE I.
Analyses of Adenine and Its Salts.

	Found.	Calculated.
	<i>per cent</i>	<i>per cent</i>
Nitrogen in free base, $C_6H_5N_5$	51.9	51.8
Sulfuric acid in sulfate, $(C_6H_5N_5)_2H_2SO_4$	26.5	26.6
Nitrogen in sulfate, $(C_6H_5N_5)_2H_2SO_4$	37.6	38.0
Water " " $(C_6H_5N_5)_2H_2SO_4 \cdot 2H_2O$	8.96	8.91
Nitrogen in picrate, $C_6H_5N_5 \cdot C_6H_3N_3O_7$	30.6	30.77
Picric acid in picrate, $C_6H_5N_5 \cdot C_6H_3N_3O_7$	62.9	62.9
Water in picrate, $C_6H_5N_5 \cdot C_6H_3N_3O_7 \cdot H_2O$	4.73	4.72
" " chloride, $C_6H_5N_5HCl \cdot \frac{1}{2} H_2O$	5.5	6.25
Sulfur: nitrogen ratio in sulfate.....	1:10	1:10

plates forming aggregates in which the plates are joined at their corners. It contains 2 molecules of water of crystallization which are not readily given off below 120° . On heating, decomposition occurs above 250° without melting.

The chloride crystallizes in thin elongated plates, often with the corners cut off, forming a rectangular point.

The picrate was prepared by precipitation of a solution of free adenine or its salt with picric acid and was recrystallized from boiling water.

The analytical data may be most concisely presented in tabular form (see Table I).

The composition of the chloride was determined by titration to faint acid reaction to Congo red. 0.1328 gm. of free base required 9.9 cc. of 0.1 N HCl. Calculated from formula $C_6H_5N_5 \cdot HCl$, 9.84 cc.

The picric acid determination was made by the nitron method (16).

SUMMARY.

The "base yielding a picrate melting at 298°" recently found in the juice of the alfalfa plant (1,2) has been identified as adenine. It is present in amounts corresponding to 0.012 per cent of the fresh plant and 0.066 per cent of the dry plant. Adenine nitrogen accounts for 2.72 per cent of the nitrogen of the "alfalfa filtrate." Adenine is therefore an important constituent of the juice which bathes the physiologically active tissue of the plant.

Attention is called to the fact that adenine picrate is described in the literature as "melting" at temperatures in the vicinity of 281°. When heated at a rate of approximately 1° in 3 seconds, pure adenine picrate decomposes, with evolution of gas, sharply at 298°.

The lower melting points previously described are attributed to the presence of a small amount of impurity which modifies the crystalline habit of the salt and also to a slow rate of heating which permits the formation of decomposition products before the characteristic gas evolution occurs. This decomposition, consequently, has taken place at a lower temperature than that herein recorded.

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THE EFFECT OF INTRAVENOUS INJECTION OF INORGANIC CHLORIDES ON THE COMPOSITION OF BLOOD AND URINE.

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These studies were undertaken for the purpose of determining the effect of intravenous injection of hypertonic solutions of the chlorides of sodium, potassium, calcium, magnesium, and ammonium on the composition of the blood and urine. The excretion of ions in the urine has been frequently studied following the ingestion of various inorganic salts (2, 6, 24, 26, 27). A review of the literature has shown that complete inorganic analyses of blood and urine, before and after injection of inorganic chlorides, have been few and the details meager. In the present experiments the ions were analyzed both in the blood and urine very soon after a given chloride was injected, and the relationship of blood concentration and urinary excretion was determined.

In the previous experiments, as a rule, large volumes of dilute solutions were injected. The amount of water introduced into the circulation was a complicating factor. By using hypertonic solutions a maximal amount of salt and a minimal amount of water could be introduced, thus eliminating the effect of large volumes of water on the circulation and a consequent flooding of the tissues and kidneys.

Sodium chloride studies were made on normal volunteers. Studies of the effect of all the salts were made on female dogs. 10 per cent solutions were used in all cases, the dosage being based on weight. Chlorine, sodium, potassium, calcium, and magnesium were estimated quantitatively in the blood serum, hemoglobin in whole blood, and chlorine, sodium, potassium, calcium, and magnesium in the urine before and after the injection of the salt

solutions. In some experiments, phosphorus and urea and ammonia nitrogen were estimated in the urine.

REVIEW OF LITERATURE.

Meyer and Cohn (25), in 1911, studied the effects of various salts of sodium, potassium, and calcium on weight and mineral balance, when added to the diets of healthy infants. They found that sodium chloride caused an increased elimination of chlorine, sodium, and potassium in the urine. Potassium was excreted in excess of the intake, resulting in a negative balance. The sodium and chlorine balances were positive. There was a gain in weight which they attributed to a retention of sodium and associated water. They found that potassium salts, as a rule, especially potassium bicarbonate, caused loss of weight and increased elimination of water, sodium, potassium, and chlorine during the experimental period. Calcium chloride had the same effects, an increased excretion of sodium and chlorine.

Miller (26) found that a sudden increase of potassium in the diet of pigs caused an increase in sodium and chlorine excretion during the following 24 hour period.

Blum, Aubel, and Hausknecht (6), in studying the effect of sodium, potassium, and calcium salts in the production of diuresis in man, found that after the administration of potassium salt, the percentage of potassium in the blood rose, while that of sodium fell. They found also that potassium salts, especially potassium chloride, caused diuresis and increased elimination of sodium. They attributed the diuresis to the sodium ion, believing it to be the regulatory agent of water exchange. The calcium salts acted in the same way, causing diuresis by increasing renal elimination of sodium.

Bogert and McKittrick (8) added 6 gm. of magnesium citrate to a diet just maintaining calcium balance in women, and found that it increased the excretion of calcium and magnesium.

Malcolm (23) observed that ingestion of soluble magnesium salts caused a loss of calcium in adult animals, while soluble calcium salts did not affect the excretion of magnesium in the same way.

Atchley, Loeb, and Benedict (2), in 1923, and Meyer and Cohn (25), in 1911, are the only authors, so far as a review of the literature shows, who report complete inorganic analysis of urine following the ingestion of calcium chloride. Atchley and his colleagues gave large doses, 20 to 35 gm. of calcium chloride each day, by mouth. Diuresis was produced. Sodium excretion was increased markedly. Calcium and potassium¹ excretion were only moderately increased.

¹ From the balance studies made during oral ingestion of calcium chloride by a patient with nephritis and edema, an increased potassium excretion was observed which could be accounted for almost entirely by the potassium in the diet.

Underhill (34) has studied the effect of feeding ammonium salts to dogs. He found that ammonium chloride caused a marked increase in ammonia nitrogen in the urine. Underhill and Goldschmidt (35) made the same observations.

Haldane (17); Haldane, Hill, and Luck (18); and Baird, Douglas, Haldane, and Priestley (3) observed that ammonium and calcium chloride caused acidosis and diuresis in normal persons.

In brief, the results of these metabolic experiments indicate that ingestion of various inorganic salts leads to increased excretion in the urine of the ions ingested, and that certain of the salts, especially those of calcium and potassium, are effective in producing diuresis with an increased elimination of sodium. All the salts, with the possible exception of calcium, cause the increased excretion of an antagonistic ion.

Greenwald (16) found that the intravenous injection of large amounts of sodium chloride into dogs did not alter the potassium content of serum.

Iversen and Hansborg (19) injected 10 per cent solutions of sodium chloride intravenously into human beings and found that the salt was deposited in the tissues so rapidly that one cycle of the circulation was sometimes sufficient to establish equilibrium between blood and tissues. Similar observations were made on sheep by Barkus (4).

Lévy (22) found that potassium and calcium chloride, given intravenously to human beings, passed quickly into the tissues, followed by a concentration of the blood which preceded diuresis.

Bock (7) injected a 1.1 per cent solution of potassium salt intravenously into rabbits at a slow rate. He observed that a moderate diuresis occurred during injection, the urine containing a high percentage of potassium and a slightly increased percentage of sodium.

In 1909, Mendel and Benedict (24) studied the excretion of magnesium and calcium after injection of various salts of these metals. They used about a 0.4 per cent solution of calcium chloride, and found that in dogs 20 per cent was eliminated in the urine the 1st day, three-fourths of which was eliminated during the first 3 hours. There was also an increased excretion of magnesium following injection of calcium, and an increased excretion of calcium and magnesium after injection of magnesium chloride. Mendel and Benedict assert that the intestines are of minor, if any, importance as a path of excretion for magnesium and calcium introduced intravenously. They do not agree with Rey (29) who found that a greater part of calcium chloride, given orally or intravenously to dogs, was excreted by the large intestine.

Denis (12) found that, after intravenous injection of calcium chloride followed by magnesium chloride, the magnesium content of the serum at the end of an hour had increased to double the normal (from 2 to 4 mg. for each 100 cc.), and remained so for an hour, when it gradually decreased. The chlorine content of the serum was not affected.

Salvesen, Hastings, and McIntosh (30) observed that intravenous injection of calcium chloride caused a considerable increase of serum calcium, dependent on the amount injected. The serum calcium returned to normal in from 3 to 5 hours.

The reports on the intravenous injection of inorganic salts indicate that sodium chloride leaves the blood stream and is rapidly deposited in the tissues. Potassium and calcium chloride act similarly. Increase of blood calcium and magnesium has been observed after injections of calcium and magnesium chloride, respectively, with a return to normal in from 3 to 5 hours.

EXPERIMENTAL PROCEDURE AND TECHNIQUE.

The 10 per cent sodium chloride solution was injected intravenously in human beings by gravity, using for the purpose a 50 cc. accurately calibrated burette with rubber tubing and adaptor attachments. The injection was made slowly, 50 cc. requiring about 7 minutes.²

Sodium chloride, 0.07 gm. for each kilo, was given. A sample of blood was drawn before the injection and another, 45 minutes after the injection in four subjects. Studies of hemoglobin, sodium, potassium, calcium, magnesium, and chlorine content were made of each sample. Enough blood was drawn also at 15 minute intervals to follow changes in hemoglobin and chlorine content. In order to standardize and control the water factor more effectively, subjects were permitted no food or water from the evening before until the expiration of the experimental period. Urine was collected for 2 hours before, and for 2 hours after, the injection. Studies were made as described.

The technique used in all salt studies on female dogs³ was as follows: The 10 per cent solutions of the inorganic chlorides were prepared as described for sodium chloride and injected with a calibrated Record syringe. A sample of blood was drawn before injection and another from 2 to 5 minutes afterward, and studied as described. Urine was collected by catheter for a period of 2 hours before injection, and for 2 hours, and in some cases 4 hours, after injection. In dogs, for each kilo of body weight, 0.1 gm. of salt was injected, and the injection was timed to 0.1 gm. each minute. No attempt was made to compare the solutions or their effects from an equimolecular view-point. The purpose, as

² By making the solutions in fresh water, distilled three times, sterilizing by autoclaving, and injecting the solutions slowly, no ill effects from the use of hypertonic solutions were encountered.

³ The dogs were not given food nor water for a period of from 12 to 18 hours before the experiment.

stated, was to study the effect of large injections of hypertonic solutions during experiments of short periods.

A single experiment was performed with each salt to compare the content of the anion and cation and hemoglobin in the blood with the simultaneous anion and cation excretion in the urine.

In general, the dogs showed little or no reaction except to calcium and magnesium chloride. In every case, vomiting followed injection of calcium chloride, and in two animals respiratory irregularity occurred after injections of magnesium chloride.

Chemical Methods.

At the beginning of the studies, the Palmer method (28) was used for the hemoglobin determinations, later, the Osgood method (27). Since the findings of the two checked accurately, the results are comparable.

For chloride determinations in plasma and serum, Smith's method (32) was used, except that the determinations were made on Folin-Wu filtrates instead of trichloroacetic acid filtrates. For chlorides in urine, the Volhard-Harvey method (36) was used.

The method of Kramer and Tisdall (21) with modifications was used for sodium in serum. Alundum crucibles were used instead of the Gooch crucibles recommended. After a preliminary packing with sodium pyroantimonate, they could be used repeatedly. A change in the method of washing was also introduced. After the crucibles had been sucked dry, the precipitate was washed twice with 1 cc. of 25 per cent alcohol and two or three times with 2 cc. of 33 per cent alcohol, care being taken to allow the crucibles to be sucked dry between washings. By using this modified technique, the determinations on serum checked very closely, usually within 4 mg.

The methods of Tisdall and Kramer (33) were used for both potassium and calcium. Magnesium in serum was determined by the method of Bogert and Plass (9) which is a combination of Kramer and Tisdall's magnesium method (20) and of Briggs' phosphorus method (11). Sodium sulfite was added to bring out the blue color, as directed by Briggs.

For determinations of sodium, potassium, and calcium in the urine, the methods of Kramer and Tisdall were used; for magnesium in the urine that of Briggs; for phosphorus in the urine that of Bell and Doisy (5); for ammonia in the urine that of Folin and Bell (14); and for urea the urease method.

For determination of the pH of the urine, Gillespie's drop indicator method (15) was used.

Normal Inorganic Values for Human Serum.

Denis and Hobson (13) give the following as normal figures for the inorganic constituents of human serum (estimated in milligrams for each 100 cc.):

Na	K	Ca	Mg	Cl
335	20.5	10	2.4	360

These agree closely with the figures of Kramer and Tisdall:

Na	K	Ca
335	19.6	9.11

The average values for the normal in the present series (Table I) were:

Na	K	Ca	Mg	Cl
340	19.0	10.8	3.0	365

Normal Inorganic Values for Dog Serum.

Denis (12) gives the following values in milligrams for each 100 cc.:

Na	Mg	Cl
294-308	1.3-2.0	340-360

Salvesen and Linder (31) give the following values:

Na	Ca	Mg
328-354	10.1-11.2	1.4-1.5

Abderhalden (1) gives the following figures:

Na	K	Mg	Cl
426	22.6	4	402.3

The average values for the normal in the present series (Table II) were:

Na	K	Ca	Mg	Cl
350	25.3	10.8	2.7	404

TABLE I.

Normal Inorganic Values for Human Serum.

Subject.	Hemoglobin.	Chlorine.	Sodium.	Potassium.	Calcium.	Magnesium.
	per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
A.	113	350	382	19.5	12.0	
B.	112	341	311	17.0	11.2	
C.	124	334	350	17.5	11.8	
D.	117	367	325	17.9	10.2	
E.		367	332	20.2	10.6	3.8
F.		355	360	19.8	10.5	3.0
G.		370	315	20.9	10.2	3.0
H.		375	360	19.5	11.0	
I.		339	345	18.5	10.4	2.6
J.		354	325	17.8	10.1	2.6
Average		365	340	19.0	10.8	3.0

TABLE II.

Normal Inorganic Values for Dog Serum.

Dog No.	Hemoglobin.	Chlorine.	Sodium.	Potassium.	Calcium.	Magnesium.
	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
G. 175	144	418	333	27.0	11.5	2.5
				Filtrate 20.3.		
G. 175	110	393	360	24.1	10.2	2.5
				Filtrate 22.2.		
G. 605		408	431	25.9		
G. 606		408	323	25.6	11.6	
G. 615	123	411	357	18.6		2.4
G. 616	101	420	356	35.3	11.5	3.0
G. 617	158	420	410	15.3	10.6	2.9
G. 624	117	406	380	25.9	9.2	2.3
				Filtrate 20.6.		
G. 175	126	415		23.9		
G. 605		378	435	25.9	11.3	4.5
G. 615	125	438	359	27.0	11.3	3.5
G. 618	95	390	310	20.3	11.0	2.9
G. 425	112	399	317	24.2	11.6	2.3
				Filtrate 20.2.		
G. 925	111	417			12.7	2.6
Black.	139	378	343	25.3	10.9	2.5
				Filtrate 20.5.		
G. 925	108	388	357			
G. 619	107	414	308	23.0	10.6	2.6
G. 610	100	383	305	31.8	11.1	2.2
G. 925	103	418				2.2
G. 610	114	390	285	31.6	10.8	3.0
G. 175	137	408	334	24.6	11.7	2.2
Average.....		404	350	25.3	10.8	2.7
				Filtrate 21.7 (serum same as filtrates 25.3).		

RESULTS.

Control experiments were run on two dogs to determine the variations in the inorganic constituents excreted in the urine in 2 hour periods. Table III shows that there is not a very great

difference in the constituents in 2 hour periods. The greatest variation occurred in potassium, and will be discussed later.

It will be noted that there may be an immediate rise in hemoglobin or a sudden fall (Table IV). In every case a fall occurs later. The behavior of hemoglobin following the injection of magnesium chloride is different from the others, in that there is always an immediate rise followed by a gradual drop to normal in 3 hours (Fig. 1). There is no appreciable change in chlorine in the blood (Fig. 2). If a change does occur, it is most marked with calcium chloride and consists in an immediate rise followed by a fall and return to normal in approximately an hour.

TABLE III.

Variations in Inorganic Values in Urine of Dogs for 2 Hour Periods.

Dog No. Weight.	Time.	Volume.	pH	Chlorine.		Sodium.		Potassium.		Calcium.		Mag- nesium.		Ammonia nitrogen.	
				Concentra- tion.	Total.	Concentra- tion.	Total.	Concentra- tion.	Total.	Concentra- tion.	Total.	Concentra- tion.	Total.	Concentra- tion.	Total.
				mg. per cent	mg.	mg. per cent	mg.	mg. per cent	mg.	mg. per cent	mg.	mg. per cent	mg.	mg. per cent	mg.
G. 175	2	7	7.2	720	50	131	9.2	929	66	9.4	0.66	27.7	2.0		
11.99	2	9	7.2	660	54	126	11.3	1,254	113	4.5	0.41	29.4	2.6		
kilos.	2	9		540	50	136	12.2	1,128	102	3.6	0.32	33.3	2.9		
G. 175	2	6	6.4	240	21	42	2.5	573	34	3.3	0.19	38.1	2.3	17	1.1
10.82	2	5	6.2	180	12	63	3.2	693	35	2.3	0.12	32.0	1.6	18	0.93
kilos.	2	5	6.2	180	12			810	41	2.0	0.1	29.4	1.5	19	0.98

There was a variation in the behavior of the cations in the serum (Fig. 3). In the case of calcium and magnesium there was a sudden decided rise followed by a gradual fall, with a return to normal in about 4 hours. Bowler and Walters (10) have obtained a curve similar to the one shown here, after intravenous injection of 10 per cent calcium chloride. In the case of sodium, there was a sudden rise and fall, with a return to normal in half an hour. Potassium showed no significant change.

There was considerable variation in the rate of excretion of the injected ions, in their diuretic effect, and in the percentage of change in the total excretion of ions over the normal period. The

pH of the urine was not affected except that after the injection of calcium and ammonium chloride, the urine became more acid (pH 5.4 to 5.0). Tables V and VI show that the salts may be arranged in a definite order as to diuretic effect, as to percentage of increase in excretion of injected ions, and as to rapidity of excretion of the injected ions. The order remains practically the same for the three processes when determined for short periods;

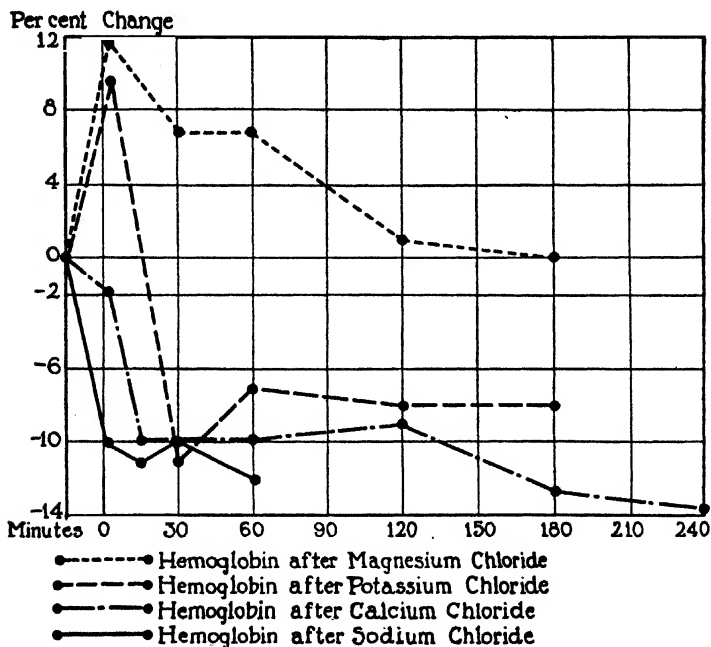


FIG. 1. Comparison of the hemoglobin of the blood after intravenous injections of 10 per cent solutions of magnesium chloride, potassium chloride, sodium chloride, and calcium chloride; 0.1 gm. for each kilo.

beginning with the greatest, it is: magnesium chloride, potassium chloride, sodium chloride, and calcium chloride (Figs. 4, 5, 6, 7, and 8).

Magnesium Chloride.—Analysis of the blood (Table VII) after the injection of magnesium chloride showed a slight rise in the chlorine content (1 to 11 per cent) of the serum. The rise in magnesium was high, varying from 290 to 638 per cent. There was no significant change in the other ions.

TABLE IV.
Blood Changes after Injection of Chlorides.

Dog No.	Salt injected.	Time. min.	Hemoglo- bin.	Change.* per cent	Anion (Cl).	Change.* per cent	Cation.	Change.* per cent	Magnesium. mg. per cent
G. 925	Sodium chloride.	Before injection.	108		388		357		
		After injection of 10 min.							
		2	97	-10	392	+1.00	448	+26	
		15	96	-11	400	+3.0	455	+26	
		30	97	-10	396	+2.0	351	-2	
		60	95	-12	387	-0.2	345	-3	
		120			387	-0.2	360	+1	
G. 175	Potassium chloride.	180			393	+1.3	365	+2	
		Before injection.	126		415		23.9		
		After injection of 10 min.							
		2	138	+9.5	422	+1.7	23.5	-1.6	
		15							
		30	112	-11.0	415	0	23.6	-1.2	
		60	117	-7.1	418	+0.7	22.6	-5.4	
G. 925	Calcium chloride.	120	116	-8.0	420	+1.2	21.6	-9.5	
		180	116	-8.0	419	+0.9	23.6	-1.2	
		Before injection.	111		417		12.7		2.6
		After injection of 10 min.							
		2	109	-1.8	437	+4.8	37.1	+192.1	2.6
		15	100	-9.9	445	+6.7	31.9	+151.1	2.6
		30	100	-9.9	463	+11.0	22.2	+74.8	2.6

Magnesium chloride had a decided diuretic effect (Table VI and Fig. 5), increasing the urine volume 350 to 460 per cent. Diuresis occurred in every experiment, being at its height during the 1st and 2nd hours after injection. The chlorine rose in concentration and total excretion. The total output, being dependent on the volume of urine, fell during the second 2 hours, but the concentration remained high. The total percentage of chlorine excreted was as much as 81 per cent in 4 hours. In the same experiment approximately 13 per cent of the injected magnesium

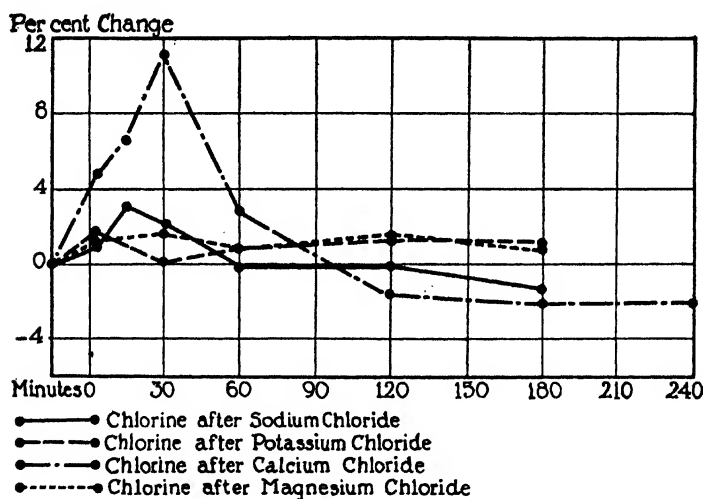


FIG. 2. Comparison of the chlorine content of the blood serum after intravenous injections of 10 per cent solutions of magnesium chloride, potassium chloride, sodium chloride, and calcium chloride; 0.1 gm. for each kilo.

was excreted in the same length of time. This chlorine-magnesium ratio varied in the different experiments. The calcium excretion was decidedly increased by magnesium.

Potassium Chloride.—In Table VII and Fig. 6 is shown the effect of the intravenous injection of potassium chloride on the blood. It will be observed that there was no appreciable rise in chlorine (average 2 per cent). Practically all the added chlorine had left the circulation in from 2 to 5 minutes after its injection. In the other experiments the rise in potassium showed a variation from

1 to 30 per cent (average 15 per cent). The other cations were not decidedly affected. In only one experiment was there a fall in serum sodium (380 to 330 mg.). In the remaining five there was either no change or a slight rise. These results are contrary to the definite decrease in serum sodium reported by Blum, Aubel, and Hausknecht (6). It was suggested that some of the injected potassium might be passing into the red blood cells, and

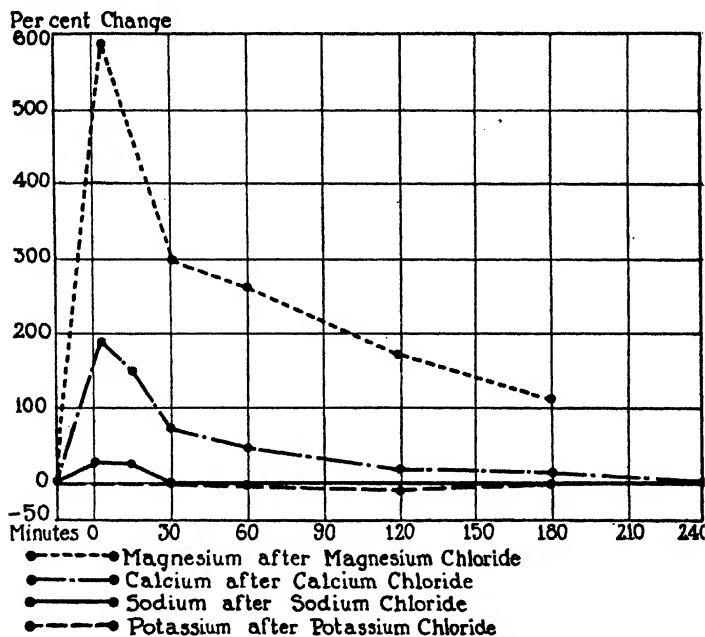


Fig. 3. Comparison of the cations in the blood serum after intravenous injections of 10 per cent solutions of magnesium chloride, potassium chloride, sodium chloride, and calcium chloride; 0.1 gm. for each kilo.

thus not leaving the circulation, as my data would tend to suggest. Consequently, in two experiments the potassium of whole blood was determined before and after injection; in one a rise of no significance (within the error of the method) occurred, and in the other, there was an increase of potassium in both serum (30 per cent) and whole blood (18 per cent). The following data and calculations in the latter experiment show that the normal ratio of potassium to serum and red cells was maintained: hemato-

TABLE V.
Urinæy Change₀₀ after Injection of Chloride₀₀.

Dog No. Weight.	Amount of salt injected.	Time.	Volume.	pH	Chlorine.					Cation.				
					Concentration.	Total.	Change.	Time.	Injected chloride excreted.*	Concentration.	Total.	Change.	Time.	Injected sodium excreted.*
	gm.	hrs.	cc.		mg. per cent	mg.	per cent	hrs.	mg. per cent	mg.	per cent	hrs.	mg. per cent	
G. 925 13.75 kilos.	Sodium chloride. 1.33	Before injection.												
		1	4.1	5.2	150	6.5				9.6	0.4			
		After injection.												
		1	3.6	5.2	300	11.0	+70	1	0.6	85	2.9	+625	1	0.46
		2	5.5	5.4	840	48.0	+688	2	5.8	88.1	4.9	+1,125	2	1.3
G. 175 11.1 kilos.	Potassium chloride. 1.1	3	7.5	5.2	960	72.0	+1,100	3	14.1	118	8.9	+2,125	3	2.9
		Before injection.												
		1	3.3	7.0	360	12				190	6.3			
		After injection.												
		1	9.7	6.8	1,270	123	+925	1	20.9	186	19.1	+203	1	2.24
G. 925 14.21 kilos.	Calcium chloride. 1.43	2	8.0	7.0	1,320	180	+1,400	2	52.6	204	16.3	+160	2	4.07
		3	6.5	7.0	1,200	78	+550	3	66.2	297	20.8	+231	3	6.5
		Before injection.												
		1	7.2	5.4	840	60				40.2	2.87			

	After injection.	3.2	5.4	540	17	-72		73.8	2.34	-18		
	1	6.0	5.0	790	47	-21		212.3	12.7	+339	2	1.8
	2	6.9	5.0	1,520	105	+75	3	161.2	11.1	+275	3	3.4
	3	6.2	5.0	1,050	61	+1.6	4	118.3	7.3	+152	4	5.2
	4											
G. 925	Before injection.											
13.2	1	4.9	5.6	690	34			37.5	1.86			
kilos.	After injection.											
	1	30.8	5.4	1,204	371	+991	1	178	55.1	+2,508	1	14.8
	2	14	5.6	1,410	192	+470	2	259	36.3	+1,850	2	24.3
	3	11	5.6	1,340	150	+341	3	257	28.4	+1,427	3	30.1

* This figure denotes the percentage of injected chloride excreted for the total period after injection.

TABLE VI.—Typical Findings in the Urine before and

after Injection of the Inorganic Chlorides.

Dog No.	Salt injected.	Time.	Chlorine.			Sodium.			Potassium.			Calcium.			Magnesium.			Ammonia nitrogen.			Phosphorus.			Injected ion excreted.		
			Concen- tration	Total	Change	Concen- tration	Total	Change	Concen- tration	Total	Change	Concen- tration	Total	Change	Concen- tration	Total	Change	Concen- tration	Total	Change	Time.	per cent	Antion.	per cent	Concn.	
G. 610	Magnesium chloride.	11 a.m. to 1 p.m.*	67.4	240 14		90 5		696 42				8.4 0.5		26 1.5							1 p.m. to 3 p.m.	67	12.1			
		1 p.m. to 3 p.m.	417.6	1,078 442	+3,057	125 51	+920	142 58	+42			28.0 11.5	+2,200	66 27.2	+1,713						1 p.m. to 5 p.m.	81.4	13.5			
		3 p.m. to 5 p.m.	117.4	824 91	+550	28 3	-40	1,560 172	+311			2.4 0.3	-40	38 4.2	+180											
		9 a.m. to 11 a.m.	46.8	270 9.6		150 2.4		1,115 50													11 a.m. to 1 p.m.	28	21.8			
G. 624	Potassium chloride.	11 a.m. to 1 p.m.	117.4	930 113	+1,077	67 7.4	+208	1,246 137	+174											11 a.m. to 3 p.m.	36	20.0				
		1 p.m. to 3 p.m.	47.2	990 40	+300	69 1.4	-41	1,005 43	-15																	
		3 p.m. to 5 p.m.	11	300 33		512 56		50 6				7.5 0.83		26 2.9												
		11 a.m. to 1 p.m.	49	912 447	+1,224	186 92	+64	1,020 500	+8,233			2.8 1.4	+70	5 2.0	-10						1 p.m. to 3 p.m.	53	58			
Black.	Sodium chloride.	3 p.m. to 5 p.m.	16	960 154	+368	99 16	-71	900 144	+2,300			4.8 0.77	-7	10 1.7	-40					1 p.m. to 5 p.m.	63	74				
		11 a.m. to 1 p.m.	77.3	60 4.2		73 5		1,392 97				6.0 0.42		21.6 1.5						1 p.m. to 3 p.m.	14	7				
		1 p.m. to 3 p.m.	147.4	540 78	+1,750	214 30	+500	1,214 170	+75			7.0 0.98	+133	33.8 4.7	+218					1 p.m. to 5 p.m.	21	12				
		3 p.m. to 5 p.m.	97.4	420 38	+600	232 21	+320	1,506 136	+40			6.6 0.60	+42	40.8 3.7	+146					12 m. to 2 p.m.	9.9	0.5				
G. 425	Calcium chloride.	10 a.m. to 12 m.	135.6	180 19		28 4		426 55				12.6 1.6		10.8 1.4						12 m. to 4 p.m.	24.1	1.1				
		12 m. to 2 p.m.	165.2	690 111	+485	47 8	+100	648 104	+90			26 4.1	+156	13.8 2.2	+57											
		2 p.m. to 4 p.m.	185.4	840 151	+695	17 3	-25	838 151	+174			26 4.7	+194	12.1 2.2	+57											
G. 175	Ammonium chloride.	12 m. to 2 p.m.																								
		2 p.m. to 4 p.m.	97.2	660 59		151 14		342 31				5.1 0.5		31 2.8							2 p.m. to 4 p.m.	7				
		4 p.m. to 6 p.m.	166.8	750 120	+103	148 24	+74	926 148	+377			5.6 0.9	+80	44 7.0	+150						2 p.m. to 6 p.m.	10				
			96.2	660 86	+45	117 11	-21	240 22	-29			18.0 1.6	+220													

* The salt was injected in each instance at the end of the first 2 hour period.

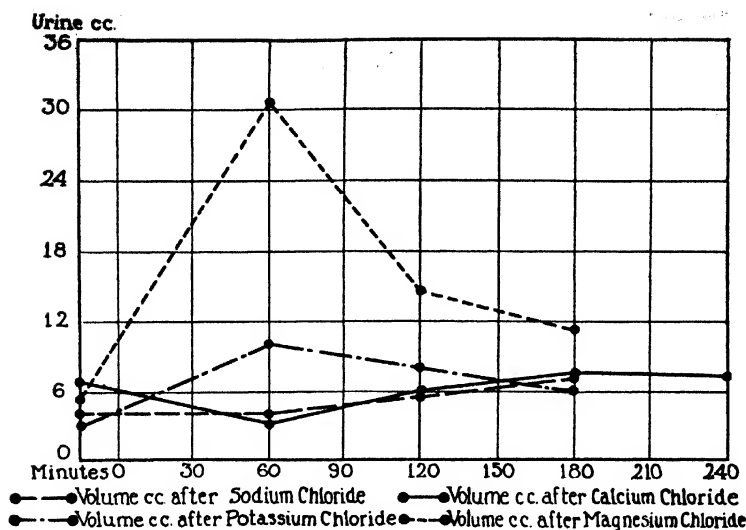


FIG. 4. Comparison of the diuresis produced by intravenous injections of 10 per cent solutions of magnesium chloride, potassium chloride, sodium chloride, and calcium chloride; 0.1 gm. for each kilo.

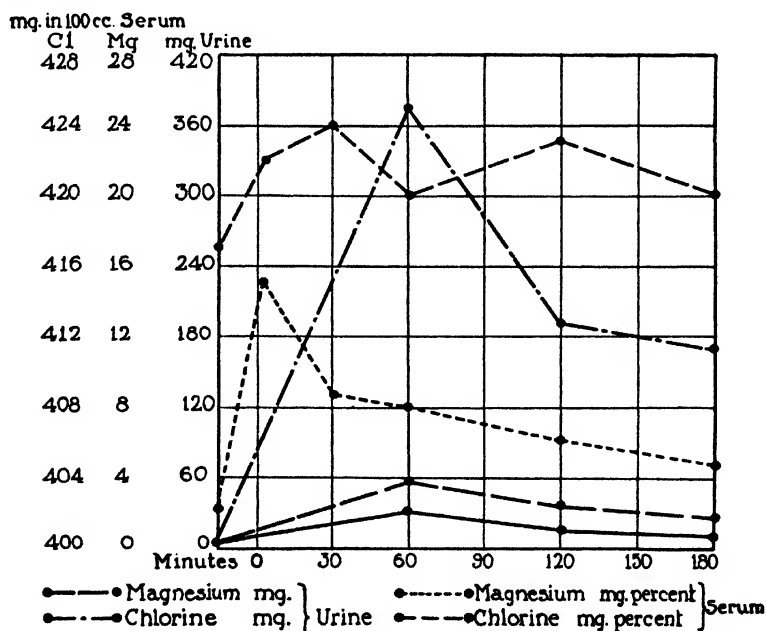


FIG. 5. Findings in the blood serum and urine following intravenous injection of 10 per cent magnesium chloride; 0.1 gm. for each kilo.

crit, erythrocytes 43 per cent, plasma or serum 57 per cent; 1 cc. of serum contained 0.3 per cent increase in potassium; 57 cc. of serum contained 17.1 per cent increase in potassium, which is practically the value obtained for whole blood. It is clear, therefore, that the potassium has not passed into the erythrocytes but left the circulation.

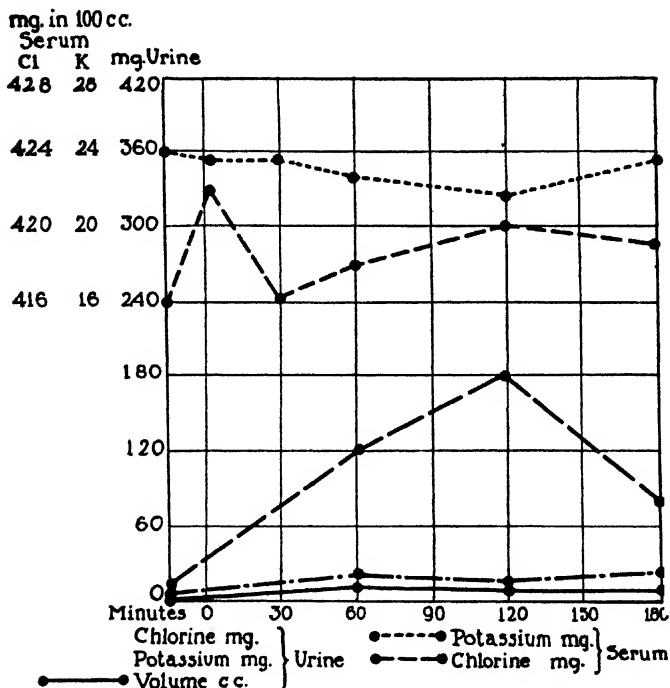


FIG. 6. Findings in the blood serum and urine following intravenous injection of 10 per cent potassium chloride; 0.1 gm. for each kilo.

Potassium chloride produced diuresis in four of five experiments (Table VI). Chlorine rose both in concentration and total output. Sodium almost doubled in total excretion, but the concentration fell except in the experiment in which no diuresis was produced. In that case the concentration rose definitely. The total output of potassium rose in every case, and the concentration also usually rose. The calcium, magnesium, and phosphorus did not seem to be much affected as to total output. The

concentration fell when the diuresis was produced so that the total excretion remained about the same.

In two experiments urine was collected at 2 hour intervals for 4 hours after the injection. At the end of 4 hours the urine volume had fallen practically to normal, but the chlorine concentration remained high. There was a fall in the total excretion of sodium

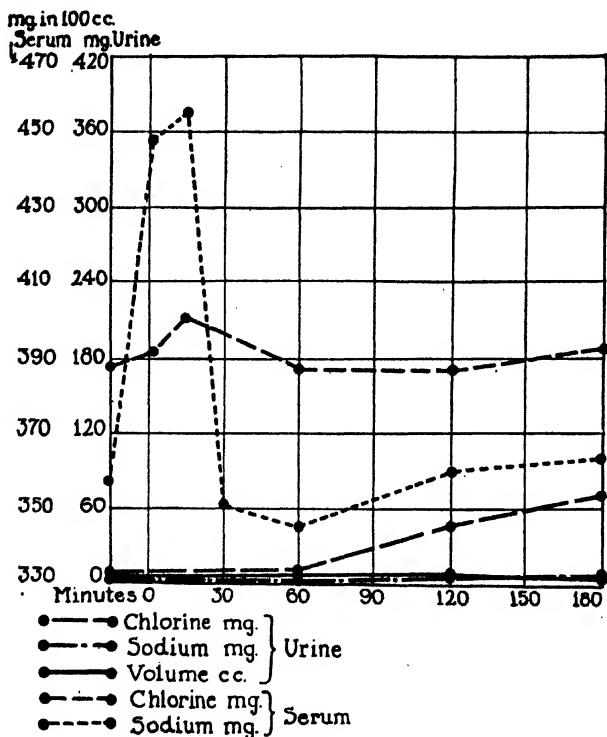


FIG. 7. Findings in the blood serum and urine following intravenous injection of 10 per cent sodium chloride; 0.1 gm. for each kilo.

and potassium. The excretion of sodium fell considerably below normal.

Sodium Chloride.—Table VII and Fig. 7 show that there was a slight rise in serum sodium and chlorine in dogs immediately after the injection of sodium chloride, but that there was no change in the other ions. In the urine (Table VI), diuresis resulted in some

cases but not in others. There was an increased excretion of sodium, potassium, and chlorine (Fig. 8).

A comparison of the effects of sodium chloride injections in man and in dogs shows that the increased excretion of potassium is a

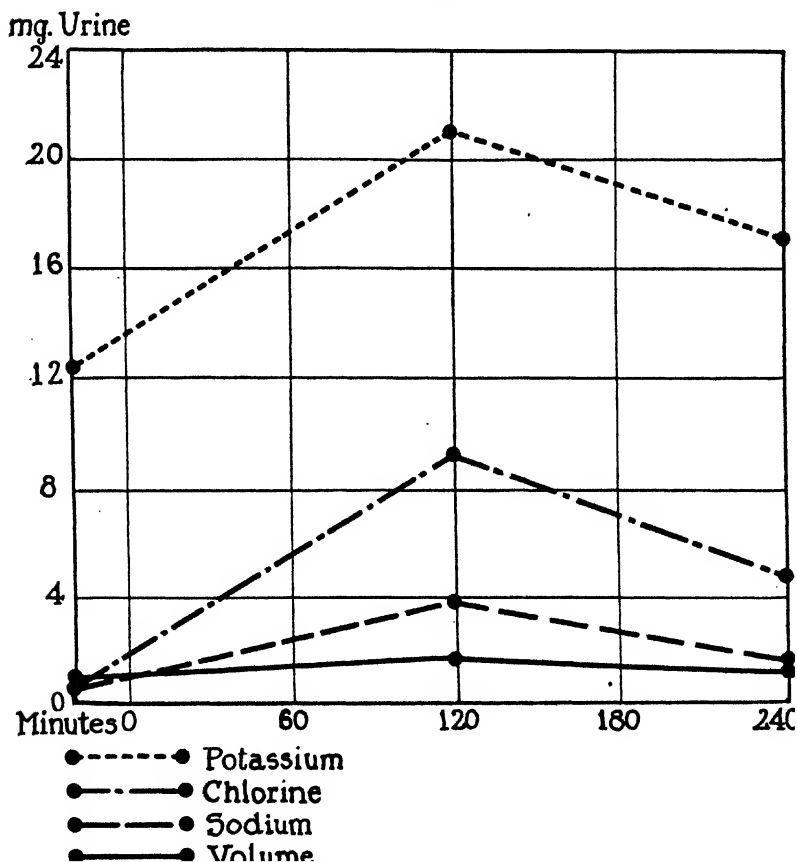


FIG. 8. Urinary findings after intravenous injection of 10 per cent sodium chloride; 0.1 gm. for each kilo.

little less marked in dogs than in man. Dogs seem to show a greater percentage of change in the excretion of calcium and magnesium.

The results of sodium chloride studies on the blood in man are given in Table VIII. It will be noticed that there was a sudden

'ABI
Blood Changes after Intravenous Injections of the Inorganic Chlorides.

Dog No. Weight.	Salt injected.	Time.	Hemoglobin.	Chlorine.	Change in anion.	Sodium.	Potassium.	Calcium.	Magnesium.	Change in cation.	Hr./thromocytes.	Remarks.
kg.			per cent	mg. per cent	per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	per cent	per cent	
G. 619 7.8	Magnesium chloride.	Before.*	107	414		308	23	10.6	2.6			Vomited; shallow, slow res- piration. Shallow, slow respiration; re- suscitation. No respiratory abnormalities.
G. 610 10.15		After.†		444	+7	340	23	11.0	19.2	+638		
G. 610 10.15		Before.	100	383		305	31.8	11.1	2.2			
G. 925 13.2		After.		426	+11	280	27.0	11.8	8.6	+290		
		Before.	102.9	418					2.2			Whole blood, 24.4. " " 25.4, 4 per cent change. Whole blood, 16.8. " " 20.0, 18 per cent change.
		After.	115	422	+1.2				15.1	+586		
G. 605 6.88	Potassium chloride.	Before.		408		431	25.9					
G. 606 5.74		After.		420	+3	462	32.6	11.2		+26		
G. 615 8.8		Before.		408		323	25.6	11.6				
G. 616 10.56		After.		420	+3	328	27.1	11.4		+6		
		Before.	123	411		357	18.6		2.4			
		After.	117	414	+0.7	358	22.4	10.4	2.4	+20		
		Before.	101	420		356	35.3	11.5	3.0		42.2	Whole blood, 24.4. " " 25.4, 4 per cent change. Whole blood, 16.8. " " 20.0, 18 per cent change.
		After.		432	+2.7	361	38.5	11.6	3.0	+6		
G. 617 15.2		Before.	118	420		410	15.3	10.6	2.9			
		After.		426	+1.0	430	20.0	12.9	2.9	+30	43.7	

G. 624 7.72	Before. After.	117 406 412		+1.2	380 330	25.9 26.0	9.2 8.9	2.3 2.4	+3	
G. 175 11.0	Before. After.	126 138 415 422		+1.7	337 448	23.9 23.5			+1.6	
Black. 8.4	Before. After.	139 378 418	Sodium chloride.	+10	343 390	25.3 25.6	10.9 11.6	3.5 2.5	+10.8	
G. 925 13.75	Before. After.	108 97 388 392		+1.0	357 448				+25	
G. 610 11.05	Before. After.	114 390 414	Ammonium chloride.	+6.0	285 273	31.6	10.8	3.0		
G. 175 12.65	Before. After.	137 408 456		+11	334 317	24.6 25.0	11.7 11.1	2.2 2.2		
G. 605 6.98	Before. After.	378 364	Calcium chloride.	-4	435 438	25.9 24.1	11.3 28.6	4.6 4.1	+153	Vomited four times during injection.
G. 615 8.43	Before. After.	125 438 450		+2.0	359 381	27.0 27.7	11.3 20.2	3.5 3.4	+79	Vomited. " 2 to 3 min. after injection.
G. 618 11.02	Before. After.	95 396 414		+6.0	310 335	20.3 20.7	11.0 15.0	2.9 2.9	+36	Vomited during injection and once 10 min. after.
G. 425 14.25	Before. After.	112 409 417		+3.0	317 340	24.2 24.9	11.6 24.9	2.3 2.3	+114	Vomited bile 2 times after injection.
G. 925 14.2	Before. After.	109 437		+4.8			12.7 37.1	2.6 2.6	+192	

* Before injection.

† After injection, which in each case took 10 minutes. The second blood was drawn from 1 to 3 minutes after the injection was completed.

drop in the hemoglobin followed by a gradual rise, the normal being reached about 1 hour after injection. The plasma chlorides either rose or fell.

It will be noted (Table IX) that after the injection of sodium chloride in normal persons, diuresis was quite variable, ranging

TABLE VIII.

Effect of the Intravenous Injection of Sodium Chloride on the Hemoglobin and Chlorine Content of the Blood in Man.

Case No.	Time.	Hemoglobin.	Chlorine.
	<i>min.</i>	<i>per cent</i>	<i>mg. per cent</i>
1	Before injection.	113	350
	After "		
	15	107	
	30	109	346
	45	108	341
	60	111	328
2	Before injection.	112	341
	After "		
	15	106	356
	30	111	363
	45	111	346
	60	111	380
3	Before injection.	124	344
	After "		
	15	113	334
	30	117	
	45	119	325
	60	120	369
4	Before injection.	117	370
	After "		
	15	107	386
	30	108	390
	45	111	380
	60	112	

from 0 to 64 cc. increase over the control excretion before the injection. There was a definite increase, both in the total excretion, and in the concentration, of the chlorine. It would seem that the smaller the increase in volume, the greater the increase

in concentration, and *vice versa*. This was true in seven of the ten normal cases. The potassium was increased both in concentration and total excretion, and in some cases considerably. The increase of potassium was more marked than that of sodium. There was only a small deviation in the calcium and magnesium; both total excretion and concentration showed an increase or decrease of a few milligrams in every case. The phosphorus showed such marked variations in the normal that no conclusions could be drawn.

In the case of one of the normal subjects the experimental period was continued for 6 hours after the injection. Table X and Fig. 9 show that the volume of the urine dropped below that of the preinjection period. There was an increase in the excretion of sodium, potassium, and chlorine, followed by a fall. The potassium excretion was greater than the sodium; it rose more sharply but did not return so rapidly to normal.

Studies of the inorganic constituents of the blood and urine, made before and after the intravenous injection of 10 per cent sodium chloride into patients for therapeutic purposes, have been described in detail.⁴ The technique and the methods employed were like those employed in the studies of normal individuals. The types of cases included malignant hypertension, marked arteriosclerosis and hypertension, essential hypertension and moderate arteriosclerosis, chronic nephritis without edema, chronic nephritis with edema, and nephrosis. Only general statements regarding the findings will be made here.

The inorganic constituents of the blood in the pathologic cases were, in general, normal. The chlorides, especially in cases of nephritis, were within the upper limits of normal. In a few cases of malignant hypertension there was a high concentration of sodium.

On the whole, the percentage of chlorine in the urine was decidedly lower in the cases studied than in normal subjects. The elimination of sodium, potassium, calcium, and magnesium was more nearly normal than that of chlorine. Those patients with chronic glomerulonephritis without edema were better able to concentrate the sodium ion and excrete it than those with edema.

⁴ Barrier, C. W., and Whelan, M., 1925 (in press).

TABLE IX.
Urinary Findings after Injection of Sodium Chloride in Normal Subjects.

Case No.	Amount injected.	Time, 2 hrs.	Volume.	Phosphorus.			Chlorine.				Sodium.				Potassium.			Calcium.			Magnesium.		
				Concentration.	Total.	Change.	Injected chloride excreted.	Concentration.	Total.	Change.	Injected chloride excreted.	Concentration.	Total.	Change.	Concentration.	Total.	Change.	Concentration.	Total.	Change.	Concentration.	Total.	Change.
kg.	gm.		cc.	mg. per cent	mg.	per cent	per cent	mg. per cent	mg.	per cent	per cent	mg. per cent	mg.	per cent	mg. per cent	mg.	per cent	mg. per cent	mg.	per cent	mg. per cent	mg.	per cent
1	4.5	Before.	35	900	782			155	136			86	75		6.1	5.4		6.1	5.4		7.7	6.8	
63.0		After.	90	1,038	934	+19.0	5.5	298.0	268.2	+97	7.3	104	93.6	+25	4.4	4.1	-24	4.4	4.1	-24	6.0	8.1	+19.0
2	4.2	Before.	40	900	359			168	67			105	42		16.0	6.5		16.0	6.5		12.3	4.9	
58.5		After.	92	960	883	+146	16.8	236	217	+222	8.7	91	83.7	+100	17.0	15.6	+140	17.0	15.6	+140	9.0	12.3	+151
3	5.6	Before.	35	528	186			182	64			410	143		10.8	3.8		10.8	3.8				
78.5		After.	50	786	393	+110	6.0	231	115.5	+81.0	2.4	540	270	+88	9.0	4.5	+18.0	9.0	4.5	+18.0			
4	5.6	Before.	90	1,116	1,002			225	202			374	357		3.6	7.8		3.6	7.8				
78.6		After.	90	1,230	1,107	+10.0	4.1	296	268.4	+30.0	2.8	619	557	+56	9.5	8.6	+10.2	9.5	8.6	+10.2			
5	5.0	Before.	40	552	222			225	90			585	234		16.0	7.0		16.0	7.0				
70.0		After.	94	888	834	+275	20.4	280	263	+192	9.6	607	571	+144	12.7	11.9	+70	12.7	11.9	+70			
6	5.4	Before.	95	840	798			274	261			361	343		19.2	18.1		19.2	18.1		6.7	6.3	
75.5		After.	96	1,280	1,229	+53	13.4	189	181.4	-30.2		429	410	+19.0	7.8	7.5	-59.0	7.8	7.5	-59.0	3.8	5.6	-11.2

7	5.0	Before.	80	11.6	9.3	1,080	846		302	242	+5	2.71	321,557	-22	10.1	8.1	5.4	4.3
73.0		After.	94	42.0	39.0	1,158	1,089	+28.0	8.1	372	360		438,432		11.0	10.3	7.1	9.9
																		+56.1
8	5.5	Before.	56	9.6	5.4	732	408		795	445			353,198		12.0	6.7	7.0	3.9
76.5		After.	120	11.2	20.0	930	1,116	+173	21.4	336	403	-9.2	328,394	+98	9.6	11.5	4.7	8.5
																		+53
9	4.4	Before.	32	5.5	17.6	810	258		214.2	68.5			283,90.6		30.1	9.6	18.1	5.8
61.0		After.	56	28.0	23.0	1,110	622	+141	13.8	341.6	181.5	+165	431,241	+168	12.8	7.2	8.1	6.9
																		-20.1
10	5.0	Before.	30	88	26	486	145.8		183.3	54.1			153,45.9		32.0	9.6	18.2	5.5
68.5		After.	40	84	50	768	307.2	+110	5.3	180.3	72.0	+33.0	472,189.0	+312	16.9	6.8	12.1	7.3
																		+32

Lévy (22) reports that sodium chloride given intravenously produced a dilution of the blood without a subsequent diuretic effect in patients kept on a salt-free diet. This may account in

TABLE X.

Urinary Findings for a Period of 6 Hours after Injection of Sodium Chloride (Case 4).

7 a.m. to 9 a.m.*	90	1,130	1,090	225	203	375	237	8.67	7.78
9 " " 11 "	90	1,750	1,106	294	265	619	578	9.54	8.6
11 " " 1 p.m.	73	1,750	913	294	212	619	492	9.54	5.34
1 p.m. " 3 "	57	1,290	720	282	158	752	427	3.6	2.08

* 5.6 gm. of sodium chloride injected at 9 a.m.

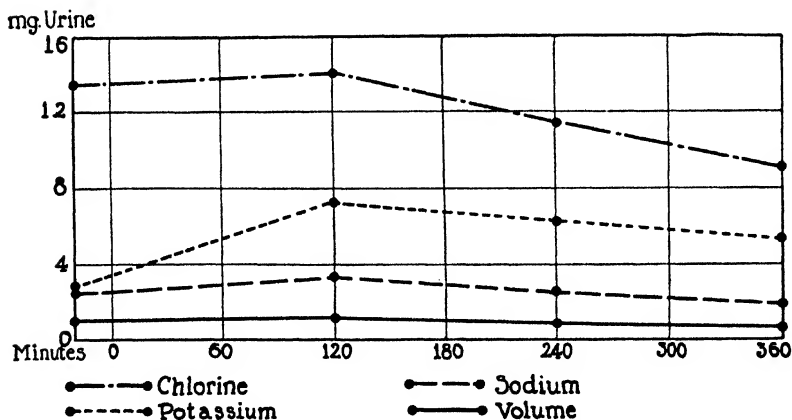


FIG. 9. Urinary findings after intravenous injection of 10 per cent sodium chloride; 0.0714 gm. for each kilo.

part for the increased concentration of sodium chloride in the urine without marked increase in the volume of urine.

Ammonium Chloride.—It was observed (Table VII) that injections of 10 per cent ammonium chloride did not cause a marked

increase of the chlorine of the blood (6 to 11 per cent) nor did it affect the other ions. In the short period of 2 hours during which observations were made, a slight increase in the volume of urine occurred (Table VI). The pH dropped definitely (7.2 to 6.2); ammonia nitrogen fell; urea nitrogen increased about 75 per cent. The chlorine increased in concentration and total

mg.in100cc.

Serum

Cl Ca mg.Urine

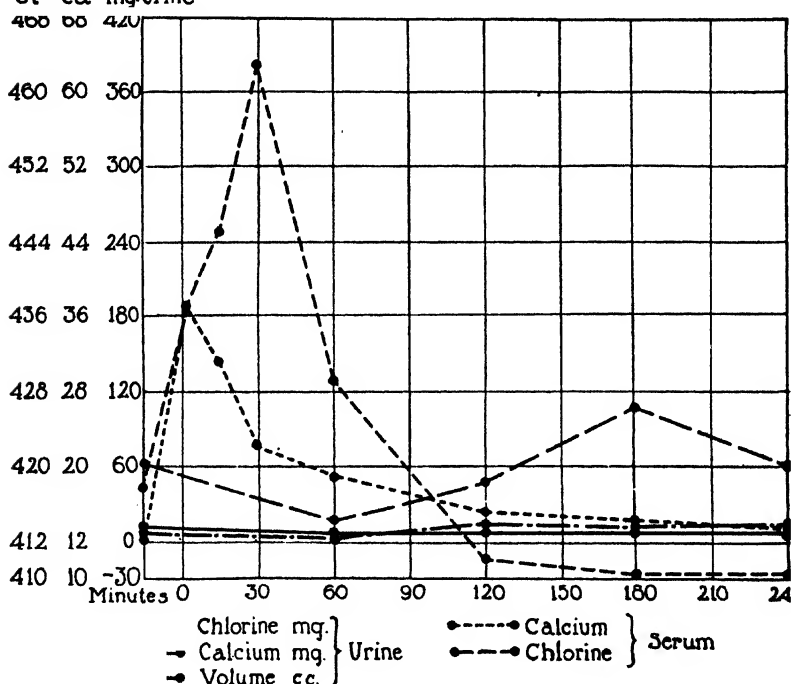


FIG. 10. Findings in the blood serum and urine following intravenous injection of 10 per cent calcium chloride; 0.1 gm. for each kilo.

excretion. There was an increased excretion of the other ions. This occurred no doubt in combination with the excess chlorine, which would seem to agree with the conclusion of Baird, Douglas, Haldane, and Priestley (3) that an acidosis followed the ingestion of ammonium chloride.

Calcium Chloride.—As shown in Table VII the rise in serum

TABLE XI.—*Comparison of the*

Dog No. Weight.	Calcium chloride in- jected.	Time, 2 hrs.	Volume.	pH	Ammonia nitrogen.			Chlorine.		
					Concentration.	Total.	Change.	Concentration.	Total.	Change.
					mg. per cent	mg.	per cent	mg. per cent	mg.	per cent
G. 618	1.24	Before.	7					240	17	
11.02		After.	18					300	55	+223
kilos.		After.*	64					450	288	
G. 368	1.24	Before.	8					446	36	
12.86										
kilos.		After.	14		5.6	0.8		410	57	+60

* 2 or 3 minutes after injection.

chlorine after the ingestion of calcium chloride is not significant (−4 to + 6 per cent). There was no change in serum potassium or magnesium, but there was a slight increase in sodium. The calcium increased about 88 to 150 per cent (Fig. 10). Results (Table VI) obtained from the analysis of the urine before and after injections of calcium chloride were extremely varied. In one case diuresis was produced and in another the excretion for the 2 hours following the injection was less than half that excreted before the injection. The rise in chlorine concentration was less marked in the former than in the latter. The pH dropped during the 2nd hour after injection. It is obvious that a slight increase in concentration accompanied by a diuresis greatly increases the total chlorine excretion. The sodium excretion was variable; the potassium was scarcely affected. Calcium excretion increased, the increase ranging from 150 to 800 per cent in 2 hours. However, the total calcium excreted in 2 hours was only 1.8 per cent (or less) of that injected. Magnesium excretion was variable. It was usually increased, but in one experiment it was decreased.

The intravenous injection of calcium chloride into dogs seemed to affect the vomiting centers. In every case the dogs vomited during or shortly after the injection. The vomitus was analyzed

in Urine, Vomitus, and Bile.

Sodium.		Potassium.			Calcium.				Magnesium.			Remarks.
Total.	Change.	Concentration.	Total.	Change.	Concentration.	Total.	Change.	Injected calcium excreted.	Concentration.	Total.	Change.	
mg.	per cent	mg. per cent	mg.	per cent	mg. per cent	mg.	per cent	per cent	mg. per cent	mg.	per cent	
6.3		515	36		10.5	0.74			33.5	2.4		
1.7	-74	440	79	+120	2.5	4.5	+509	0.48	8.0	1.4	-41	Urine. Vomitus containing bile.
140.3		44	28.2		13.7	8.8			2.3	1.5		
24		21.3	1.7		13.2	1.1			2.5	0.2		
51	+112	23.1	3.2	+88	17.8	2.5	+127	0.31	2.0	0.28	+40	Bile.

in a few instances (Table XI) and found to contain considerable bile. It was suggested that perhaps calcium chloride caused increased excretion of some of the ions into the intestine. In order to follow up this suggestion, the same dosage of calcium chloride was given intravenously to a dog with a biliary fistula and the bile collected for periods of 2 hours before and after injection. The volume of bile increased; its total content of sodium, chlorine, and calcium was doubled or nearly so; potassium and magnesium were not affected. It was found that the bile before the calcium chloride injection contained no ammonia nitrogen and afterward there was a concentration of 5.6 mg. of ammonia nitrogen for each 100 cc. of bile.

DISCUSSION.

Effect of 10 Per Cent Chloride Solutions on the Blood.—The maximal increase of chlorine in the blood was 11 per cent. All the salts, with the exception of potassium chloride, caused a variation in percentage increase of from 1 to 11 per cent. The maximal increase obtained with potassium chloride was 3 per cent. This small rise in serum chlorides is possibly explained by the smaller proportion of chlorine in potassium chloride. During the calcium

chloride experiment in which the curve of chlorine in the blood was followed, the height of chlorine concentration (11 per cent increase) was reached 30 minutes after injection, but there was a return almost to normal in an hour. When a change occurred, in dogs especially, there was an immediate rise regardless of the salt used. In sodium chloride experiments with normal human subjects, no very significant change occurred, but even the slight change was not constant, a rise occurring sometimes and a fall at other times.

With the cations, the results were different. There was an immediate rise or no change at all. Calcium and magnesium acted much alike in that there was an immediate definite rise in the serum, followed by a gradual decrease with a return to normal in approximately 4 hours. The sodium content rose definitely in these three cases, as shown in Fig. 3, and also in subsequent experiments in which the sample of blood was drawn immediately after injection, but returned to normal in 30 minutes. The potassium salts acted differently from the others. Potassium, as shown by Fig. 3 and by the other potassium chloride experiments, may, like the sodium, rise definitely in the serum, or it may show no appreciable change. Its action is much like that of sodium in that if a rise does occur it is followed by a return to normal in a relatively short time.

From the data presented here, the cations, or in fact the salts, because of the insignificant chlorine changes, can be arranged in definite order as to their disappearance from the blood serum. The order would be as follows: potassium, sodium, calcium, and magnesium. With the introduction of such an excess of salt into the blood stream, it is of interest that a definite quantitative increase of the individual cation alone occurs.

The hemoglobin changes, in the experiments in which they were followed, vary somewhat. In dogs there may be an immediate rise. This occurred with potassium and magnesium chloride. In the case of potassium chloride the initial rise was followed by a definite drop. With magnesium chloride the rise was not followed by a definite, but by a gradual, fall to normal in 3 hours. This suggests a concentration of the blood during diuresis. In all other cases, there is a drop in hemoglobin following the injection of the salts. In human subjects, the fall in hemoglobin is con-

stant and followed by a more or less gradual rise with a return to normal in about an hour. In dogs, the fall may or may not be followed by a readjustment of blood volume.

Effect on the Urine.—In the order of the diuretic action produced during a short period, the chlorides would be arranged as follows: magnesium, potassium, sodium, ammonium, and calcium. Calcium chloride is at the bottom of the list. The diuretic action, as well as the rapidity of excretion of the injected anions and cations may be delayed in the case of some of the salts, thus making their effect unappreciable during a short experimental period. The condition of the animal at the time of the injection also plays an important part. The diuresis following the injection of magnesium chloride was definite and at its height within 2 hours after injection. The diuresis occurring after the injection of the other salts varied in degree.

There was an increase in the concentration and total excretion of chlorine following all injections. The chlorine concentration remained high to the end of the experiment, but the total chlorine varied directly with the volume of the urine. The amounts of the injected ions excreted within the experimental period have been calculated, and again the previous arrangement of the salts holds true.

The excretion of the excess ions may be delayed. The disappearance from the blood and the final excretion may be dependent on many factors not readily ascertained. Much of the injected salt no doubt is deposited in the tissues and part is excreted immediately. The actual amount of the ions already present in the tissues, the balance between the individual ions, as well as the acid-base balance and water content are closely associated with the problem of the reaction of the body to injected salts.

In general, the effect of the salts on the cations excreted is peculiar to the cation injected. Calcium may or may not cause an increase in excretion of magnesium. It has a variable effect on the other cations. Sodium caused a slightly increased excretion of the other cations, the effect being greatest in the case of potassium. This is especially marked in normal human beings. Potassium affected sodium to a greater extent than the other ions. As shown by the control experiments, potassium is the most variable of the cations. During the short observation period, it showed the greatest variation.

Ammonium chloride caused an increased excretion of all cations and urea, but there was no increased excretion of ammonia nitrogen in 2 hours. Underhill (34) noticed that in dogs on a mixed diet and also in dogs in a state of inanition, there was a temporary retention of ammonia nitrogen. The increased excretion of cations was probably caused by the chlorine thus set free. In order to neutralize the excess acid the cations were combined with the chlorine and were excreted. In this way, the acidosis noted by Haldane and associates (18) may be accounted for.

Denis (12) attributes the rapid disappearance of sodium and chlorine from the blood, following injection of sodium and magnesium chloride, principally to rapid excretion by the kidney, and to the fact that magnesium does not leave the circulation as rapidly as chlorine, owing to a weaker tendency on the part of the kidney to excrete magnesium. The present work would indicate that the disappearance from the circulation is not so much dependent on the kidney as on the tendency of the tissues to take up the injected ions.

CONCLUSIONS.

1. 10 per cent solutions of the chlorides of sodium, potassium, calcium, magnesium, and ammonium may be injected intravenously into dogs without marked abnormal symptoms if the injections are carried out slowly. Amounts as large as 0.1 gm. for each kilo of body weight may be used.

2. Following the injection of these salts, the hemoglobin may show an immediate rise or an immediate fall.

3. The chlorine in the blood shows no significant quantitative changes.

4. The chlorine in the urine rises in concentration and total amount in all experiments. The concentration remains high through the experimental period. The total excretion of chlorine varies with the volume of the urine.

5. Magnesium and calcium show significant immediate rises in serum with a gradual fall, reaching normal in about 4 hours. Sodium shows an immediate rise with return to normal in $\frac{1}{2}$ hour. Potassium shows considerable variation, but is, in general, similar to sodium.

6. In dogs, according to the diuretic effect and rapidity of the

excretion of the injected ions during the short experimental period, the salts may be arranged as follows: magnesium chloride, potassium chloride, sodium chloride, ammonium chloride, and calcium chloride.

7. All of the anions and cations of the injected salts are not held together in the usual manner within the organism, and need not be excreted in combination with each other.

8. The only salt which invariably gives rise to a diuresis is magnesium chloride, and with this salt the changes in the pH of urine are insignificant. Calcium chloride always depresses the pH, while diuresis may or may not occur.

9. In dogs and man, there is a difference in the excretion of urine after the injection of sodium chloride. The concentration of potassium in dog's urine is higher normally than in that of man, and rises more sharply after injection. In man, the chlorine concentration is the higher. The curves of excretion of potassium and chlorine in dogs and man follow each other closely.

10. It would seem that the disappearance from the circulation of injected ions is dependent on many factors, among which may be the tendency of the tissues to take up the ions, and renal permeability. The first of these seems to be of greater importance.

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SAPONINS.

I. THE SAPOGENIN OBTAINED FROM SOAPNUTS.

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Although the saponins have been the subject of much investigation in the past, but little is known of their chemical structure. This work largely belongs to a period when few criteria were available for determining the homogeneity of the various substances isolated from the different plant sources. Since few of the saponins could be obtained in crystalline form, much of this work comes under suspicion, and attempts to place the substances in definite series with certain formulas were premature. However, it is well established that with but few exceptions the saponins are glucosides. Some progress has been recently made in the identification of the sugars occurring in a number of these substances; also in the preparation and preliminary study of several sapogenins, the non-carbohydrate constituents such as digitogenin, hederagenin, senegenin, albsapogenin, and others. It is recognized that the sapogenins are largely responsible for the physical and perhaps biological peculiarities of the saponins, and their further investigation is of importance.

In the present work the sapogenin of soapnuts has been made the subject for structural studies. The alcoholic extract of the shells on hydrolysis gave readily in excellent yield an apparently homogeneous crystalline substance which was easily purified. The preliminary isolation of the saponins was therefore unnecessary. The soapnuts employed were obtained in the open market, but after inquiry we are of the opinion that they were perhaps the fruit of *Sapindus saponaria* L. Several lots of different origin have yielded the same sapogenin. However, an attempt is being made to procure botanically identified material for comparison. A

sapogenin obtained from *Sapindus mukorossi* has already been described by Winterstein and Blau¹ which, however, according to the descriptions of these authors appears to be quite different from the sapogenin prepared by us, although this point is being further investigated.

In the preliminary study of the substance, the combustion of the sapogenin and some of its derivatives, performed in the usual way, gave results which were low in comparison with the analytical results obtained with certain other derivatives. Following the suggestion of van der Haar² of mixing the substance to be burned with copper oxide, consistent results were then obtained which indicated the formula $C_{31}N_{50}O_4$. Since this formula is the same as that given to hederagenin from *Hedera helix* by van der Haar² and since the *Sapindus* sapogenin had proved also to be a dihydroxy acid, it was compared directly with hederagenin prepared from ivy leaves. The results of this comparison demonstrated beyond question the identity of our sapogenin with hederagenin. Since most of the work was done before the realization of this fact, a number of derivatives had been made which had already been prepared by van der Haar from hederagenin and which, therefore, permitted a more thorough comparison. Although in some respects it may seem a duplication we are reporting these results as evidence of the identity of the substances from both sources, especially in view of what follows. In general the results here given are a confirmation of the observations reported by van der Haar, but in certain instances our results have differed in a way which is difficult to comprehend. Van der Haar,³ mentions a diacetylhederagenin which possesses a very labile acetyl group. This is readily removed by heating to 110°C. or by boiling the sub-

¹ Winterstein, E., and Blau, H., *Z. physiol. Chem.*, 1911, lxxv, 410. Winterstein, E., and Maxim, M., *Helv. Chim. Acta*, 1919, ii, 195.

² van der Haar, A. W., *Arch. Pharm.*, 1912, ccl, 430; 1913, ccli, 632; *Ber. chem. Ges.*, 1921, liv, 3142. van der Haar, A. W., and Tamburello, A., *Ber. chem. Ges.*, 1921, liv, 3148.

³ Unfortunately we were unable to obtain A. W. van der Haar's Dissertation (Bern, 1913) which possibly may have given in greater detail the preparation and properties of this substance, although the communication in *Arch. Pharm.*, 1913, ccli, 632, which is given as an essential digest of the same contains but little which permits of comparison. The preparation of the monoacetyl compound from the diacetate is described in *Ber. chem. Ges.*, 1921, liv, 3150.

stance in aqueous alcoholic solution with the formation of a crystalline monoacetyl derivative with the melting point of 156°. On the contrary we have prepared a crystalline diacetyl compound by the use of acetic anhydride which was repeatedly recrystallized from dilute alcohol or methyl alcohol without appreciable cleavage of acetic acid and which sinters at 156–159° to a vitreous mass which melts above 170°. This point has an important bearing on the interpretation of the structure of hederagenin since van der Haar has suggested that the lability of the acetyl group observed by him is due to the proximity of one of the hydroxyls to the carboxyl group. Our results do not support this view. The behavior of *Sapindus* sapogenin towards acetic acid also would seem to weaken this assumption. When boiled with this reagent the substance is acetylated and either a monoacetate or a diacetate is formed, depending upon the duration of the reaction. The diacetate proved to be identical with that formed by means of acetic anhydride. The monoacetate apparently was not identical with that described by van der Haar. If the substance in the hands of this worker was a monoacetate it is possible that the difference between the two substances is attributable to acetylation on different hydroxyl groups.

The bromobenzoates of both the sapogenin and its methyl ester here reported were found useful in deriving the formulas since the bromine values are more characteristic than the carbon and hydrogen figures. The fact that the same sapogenin occurs in such widely separated sources as *Sapindus saponaria* and *Hedera helix* is of interest, and, in all likelihood, other instances of a similar nature will be found.

EXPERIMENTAL.

Sapindus Sapogenin (Hederagenin).—500 gm. of the finely ground shells of soapnuts were extracted twice with 2 liters of 95 per cent alcohol. The combined extracts were concentrated to 1,500 cc., then treated with an equal volume of 10 per cent aqueous hydrochloric acid, and boiled under a reflux condenser. The clear, deeply colored solution at first set to a gelatinous mass, apparently of "prosapogenin." This gradually redissolved as the hydrolysis progressed and was replaced by a heavy crystalline deposit of sapogenin. After 3 hours the reaction was complete

and the mixture was allowed to cool and stand for 24 hours. The crude substance was collected and washed thoroughly with 50 per cent alcohol. The dried material was then digested at room temperature with a small volume of acetone which removed most of the highly colored impurities. For recrystallization the collected residue was dissolved in hot 60 per cent alcohol by the addition of sufficient sodium hydroxide and, while hot, reprecipitated with acetic acid. The thick gel which first formed gradually crystallized when seeded and kept hot. The yield varied from 25 to 30 gm. For final purification the still colored substance was bone-blackened in boiling alcoholic solution and the filtrate was boiled down to crystallization.

The saponenin forms colorless, well formed stout prisms and rhombic crystals which melt at 327–329° with preliminary sintering (325–326°, van der Haar). This showed no depression when mixed with hederagenin obtained from ivy leaves. In its behavior towards solvents it agrees with the properties described by van der Haar for hederagenin. It is insoluble in water and slowly but appreciably soluble in alcohol. It dissolves readily in pyridine and is either insoluble or very sparingly soluble in the other usual solvents. Although the titration in alcoholic solution showed the presence of a carboxyl group, it does not dissolve in aqueous alkali, probably due to the insolubility of its alkali salts in water. It is readily dissolved by dilute alcoholic sodium hydroxide solution, although if too concentrated, the sodium salt rapidly crystallizes. The latter is less soluble in strong alcohol than in the dilute solvent.

In sulfuric acid it forms at first a colorless solution which changes very slowly through an orange-pink to a deep purple-red. This color change is very rapid on heating. The substance does not possess a methoxyl group.

$[\alpha]_D^{21} = +80^\circ$ ($c = 2.009$ in pyridine). For hederagenin van der Haar found $[\alpha]_D^{19} = +81.2^\circ$.

$C_{31}H_{50}O_4$. Calculated. C 76.48, H 10.36.

Found. " 76.50, " 10.14.

1.5098 gm. were dissolved in 200 cc. of hot neutral alcohol, cooled, and titrated against phenolphthalein with 0.1 N NaOH. $C_{31}H_{50}O_4$. Calculated, 31.05 cc. Found, 31.35 cc.

After boiling several hours with 5 per cent sodium hydroxide in 50 per cent alcohol the sapogenin was recovered unchanged after acidification.

$$[\alpha]_D^{25} = +81^{\circ} \text{ (c = 1.009 in pyridine).}$$

Hederagenin, itself, did not yield an acetonyl derivative under the conditions used in the case of the ester, as given in the following communication.

Hederagenin Monoacetate.—5 gm. of sapogenin were refluxed for $1\frac{1}{2}$ hours in 200 cc. of glacial acetic acid. The clear solution was then concentrated under diminished pressure to dryness. The gum was dissolved in alcohol and the solution was re-concentrated to remove the excess of acetic acid. The residue formed a slowly crystallizing gum. When extracted with ether a portion remained insoluble from which unchanged hederagenin was recovered. The ethereal solution yielded a residue which was dissolved in a small volume of hot methyl alcohol. The crystals which slowly separated were collected and recrystallized from methyl alcohol, forming rosettes of delicate needles, which contained approximately 1 mol of water. The melting point was not sharp. Above 240° it slowly softened, becoming a vitreous mass at about 260° , but was not completely fluid until $270\text{--}275^{\circ}$ was reached. Van der Haar and Tamburello report a melting point of 156° and the question arises whether their substance is isomeric with ours. Our substance is readily soluble in alcohol, acetone, and ether, and but sparingly soluble in benzene.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$\text{C}_{33}\text{H}_{52}\text{O}_5 \cdot \text{H}_2\text{O}$. Calculated. H_2O 3.30.

Found. " 4.01.

$\text{C}_{33}\text{H}_{52}\text{O}_5$. Calculated. C 74.94, H 9.92.

Found. " 74.60, " 9.97.

The presence of one acetyl group was confirmed by titration after saponification. 0.1106 gm. was refluxed for 1 hour with 15 cc. of alcohol and 15 cc. of 0.1 N NaOH, and titrated back against phenolphthalein. Calculated, 4.2 cc. Found, 4.4 cc.

Hederagenin Diacetate.—5 gm. of hederagenin were refluxed in 50 cc. of acetic anhydride for 45 minutes. After decomposing the mixture with water the gum which hardened on standing was

collected and dissolved in alcohol. This solution was concentrated under diminished pressure to remove acetic acid and the residue was taken up in 70 per cent alcohol. The diacetate slowly crystallized. Recrystallized repeatedly from methyl alcohol it forms narrow platelets or stout needles which do not melt sharply. It sinters to a vitreous mass at 156–159° and does not completely melt until 170–175° is reached. It is easily soluble in the usual organic solvents. In H_2SO_4 it gives the same color as hederagenin, but it develops more slowly on heating.

Occasionally an anhydrous substance was obtained from methyl alcohol, but samples were also obtained which contained water of crystallization. This varied in different preparations from 0.5 to 1.5 mols. Contrary to the statements of van der Haar, no evidence of the cleavage of acetic acid during the recrystallization of the substance was observed or during the drying of the hydrates at 100° and 15 mm.

$$[\alpha]_D^{25} = +64^\circ \text{ (} c = 1.000 \text{ in 95 per cent alcohol).}$$

$\text{C}_{36}\text{H}_{54}\text{O}_8$. Calculated. C 73.63, H 9.54.

Found (a). " 73.49, " 9.23.

(b). " 73.85, " 9.21.

The presence of two acetyl groups was confirmed by titration after saponification. 0.1021 gm. was refluxed for 1 hour with 25 cc. of 0.1 N NaOH and 25 cc. of alcohol, and titrated back against phenolphthalein. Calculated, 5.4 cc. Found, 5.6 cc.

In another experiment 0.0974 gm. was directly titrated in alcoholic solution. Calculated, 1.72 cc. Found, 1.70 cc. An excess of 0.1 N NaOH was then added, the mixture was heated for 1 hour and then titrated back. Calculated for two acetyl groups, 3.45 cc. Found, 3.80 cc.

The diacetate was also obtained by the method used for the preparation of the monoacetate. If the acetic acid solution of the sapogenin is boiled for 3 hours the residue obtained after removal of excess acetic acid is completely soluble in ether. Fractionation from methyl alcohol, however, showed the product to be contaminated still by the monoacetate. Conversion into the diacetate was complete in 6 hours. The residue obtained after removal of the acid was dissolved in 70 per cent alcohol and allowed to crystallize. After recrystallization from methyl alcohol a diace-

tate was obtained possessing the same melting point and identical in all other respects with the substance obtained with acetic anhydride.

$C_{33}H_{44}O_6$. Calculated. C 73.63, H 9.54.
Found. " 73.39, " 9.30.

0.1031 gm. when saponified with alcoholic 0.1 N NaOH consumed 5.4 cc. Calculated for 3 equivalents, 5.40 cc.

Hederagenin Dibenzoate.⁴—5 gm. of hederagenin were benzoylated in 50 cc. of pyridine with 5 cc. of benzoyl chloride. After several hours the mixture was poured into dilute H_2SO_4 . The collected and washed amorphous precipitate was dissolved in a small volume of alcohol. After long standing crystallization began which was then facilitated by rubbing. The substance was recrystallized from alcohol. Since the separation from the relatively large volume of alcohol necessary for solution was very incomplete, concentration to smaller volume was necessary. The benzoate separated as boat-shaped platelets which melted at 290–291° after slight preliminary sintering. It is appreciably soluble in ether, benzene, and acetone, but very sparingly soluble in alcohol.

On saponification in the usual manner with an excess of alcoholic 0.1 N NaOH, 0.1027 gm. consumed 4.8 cc. Calculated, 4.45 cc.

$C_{46}H_{68}O_6$. Calculated. C 77.75, H 8.41. .
Found. " 77.25, " 8.12.

Hederagenin-Di-o-Bromobenzoate.—This was prepared as in the case of the dibenzoate. The crude amorphous product was dissolved in a necessarily large volume of hot alcohol and concentrated to small volume. Amorphous globules separated on cooling, followed on standing by rosettes of needles. Recrystallized from dry acetic ether it formed colorless needles and thin long platelets which sintered to a vitreous mass at about 165° and melted at 203–205°. It is sparingly soluble in the usual solvents.

$C_{46}H_{56}O_6Br_2$. Calculated. C 63.36, H 6.62, Br 18.75.
Found. " 63.41, " 6.48, " 18.74.

⁴ In the dissertation of van der Haar a dibenzoate is mentioned, but in view of its inaccessibility to the writer a comparison with the properties of our dibenzoate has not been possible.

Hederagenin Methyl Ester.—100 gm. of sapogenin were refluxed for several hours with a solution of 19 gm. of potassium hydroxide in 800 cc. of methyl alcohol and 36 cc. of methyl sulfate. The excess of methyl sulfate was destroyed by the further addition of an excess of alkali. The ester which crystallized on cooling was collected and after careful washing with water recrystallized from alcohol. After recrystallization from methyl alcohol it formed needles containing 1 mol of water of crystallization which melted at 238–240° after slight preliminary sintering. For hederagenin methyl ester prepared by the same and other methods, van der Haar⁵ has given 240°. When mixed with the ester prepared from ivy hederagenin no depression was noted. Characteristic for the ester obtained from both sources is the easy formation of the acetyl derivative to be described in the following communication.

$$[\alpha]_D^{21} = +75^\circ \text{ (c = 1.015 in 95 per cent alcohol).}$$

$$[\alpha]_D^{22} = +76^\circ \text{ (c = 1.030 in acetone).}$$

Air-Dry Substance. Dried at 100° and 15 mm. over H₂SO₄.

$$\text{C}_{32}\text{H}_{52}\text{O}_4 \cdot \text{H}_2\text{O}. \text{ Calculated. } \text{H}_2\text{O}, 3.47.$$

$$\text{Found. } \quad \quad \quad \text{“ } 2.99.$$

Anhydrous Substance.

$$\text{C}_{32}\text{H}_{52}\text{O}_4. \text{ Calculated. } \text{C } 75.73, \text{ H } 10.48, \text{ OCH}_3, 6.20.$$

$$\text{Found (a). } \quad \quad \quad \text{“ } 76.45, \text{ “ } 10.43.$$

$$\text{(b). } \quad \quad \quad \text{“ } 76.67, \text{ “ } 10.47.$$

$$\text{(c). } \quad \quad \quad \text{“ } 6.76.$$

Unsuccessful attempts to recover the sapogenin from the ester by long continued boiling in 10 per cent alcoholic potassium hydroxide gave evidence of the resistance of the ester to saponification. When the ester was shaken in acetic acid solution with hydrogen and an active palladium black no absorption was noted. If a solution of the ester in dry acetone is treated with 10 per cent methyl alcoholic potassium hydroxide a turbidity is produced followed by the deposition of needles of a potassium “salt.” This was obviously not a true salt but perhaps a molecular compound with either potassium hydroxide or methylate since, when filtered off, it was strongly alkaline. If dissolved in alcohol and the solution treated with water the unchanged ester was recovered, showing the ready dissociation of the compound. Its instability rendered difficult its preparation for analysis.

⁵ van der Haar, A. W., and Tamburello, A., *Ber. chem. Ges.*, 1921, liv, 3152.

Hederagenin Methyl Ester Diacetate.—This was prepared with acetic anhydride and also by dissolving the ester in acetic acid containing dry hydrochloric acid. It formed stout needles and long platelets from methyl alcohol which melted at 190–193°. Van der Haar² gives 193°. Saponification showed the presence of two acetyl groups and yielded hederagenin methyl ester.

$C_{28}H_{48}O_6$. Calculated. C 73.91, H 9.66.
Found. " 73.45, " 9.53.

Hederagenin Methyl Ester Di-o-Bromobenzoate.—Prepared from *o*-bromobenzoyl chloride and the ester in pyridine solution the compound when recrystallized from absolute alcohol formed colorless delicate needles which melted at 205–206° with slight preliminary softening. It is soluble in acetone, benzene, and ether, and but sparingly so in alcohol.

$C_{40}H_{40}O_6Br_2$. Calculated. C 63.74, H 6.75, Br 18.46.
Found. " 63.84, " 6.74, " 18.90.

SAPONINS.

II. ON THE STRUCTURE OF HEDERAGENIN.

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The results of the preceding communication are a confirmation of the conclusions of van der Haar that hederagenin is a dihydroxy acid with the formula $C_{31}H_{50}O_4$. The failure of attempts to reduce the ester with palladium and hydrogen would seem to indicate the saturated character of the basic hydrocarbon, $C_{31}H_{52}$, from which it is derived. If this is so, hederagenin would consist of six carbon rings. However, such a conclusion is perhaps premature, since the failure of this mode of hydrogenation does not always exclude the presence of double bonds. In the present investigations further information with regard to the structure of hederagenin has been obtained.

If this substance is treated with thionyl chloride not only is the carboxyl group converted into the acid chloride but, simultaneously, the hydroxyl groups are esterified with the formation of a neutral sulfite in which one SO group bridges the two hydroxyl groups. When this chloride was boiled with methyl alcohol a methyl ester was obtained which proved to be the neutral sulfite of hederagenin methyl ester, since, when saponified, it yielded hederagenin methyl ester and sulfur dioxide.

Van der Haar¹ has also reported the use of thionyl chloride for the preparation of what he considered to be the chloride of hederagenin. Without attempting its purification it was used by him directly for the preparation of what he likewise considered to be hederagenin methyl ester and hederagenin amide. However, since the melting points of the simple methyl ester and its sulfite are practically identical, it seems likely that he has confused the

¹ van der Haar, A. W., and Tamburello, A., *Ber. chem. Ges.*, 1921, liv, 3152, 3157.

material in hand with hederagenin methyl ester. The same has been found to be true for the amide prepared from this chloride. Hederagenin amide was prepared by saponifying the sulfite.

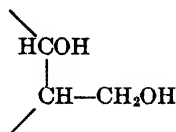
The formation of the sulfite indicates the proximity of the hydroxyl groups in hederagenin. This conclusion was verified by the preparation of an acetonyl derivative of hederagenin methyl ester, which was formed with remarkable facility. When the latter was dissolved even in ordinary acetone and then treated with a few drops of hydrochloric acid there was an immediate separation of the sparingly soluble acetonyl compound. This derivative did not react with acetic anhydride and readily yielded the original methyl ester on hydrolysis. From these facts it may be concluded that the hydroxyls are either on adjacent carbon atoms or on those which are removed by one as in trimethylene glycol. The results of the following oxidation experiments are more in harmony with the latter assumption.

Hederagenin methyl ester was oxidized in acetone solution by permanganate. The filtrate from manganese dioxide yielded a substance which analysis showed to possess the formula $C_{32}H_{50}O_4$. This substance which we have called *hederagonic methyl ester* yielded an oxime and a monoacyl compound, an *o*-bromobenzoate. It did not reduce Fehling's solution or reduce ammoniacal silver solution properties usually possessed by α -hydroxyketones. The carbonyl group therefore contained in the hydroxyketone is not adjacent to the hydroxyl group.

On extraction of the MnO_2 precipitate with alcohol, a sparingly soluble potassium salt of an acid was obtained which, from the analysis, titration, and later cleavage into its components, proved to be a molecular addition product of hederagenin methyl ester and an acid, $C_{32}H_{50}O_5$. This compound, $C_{32}H_{52}O_4 \cdot C_{32}H_{50}O_5$, was stable not only as the salt but as the acid which could be repeatedly recrystallized without decomposition. On attempting to acylate it, however, the diacyl compound of hederagenin methyl ester was all that could be recovered. The cleavage of the complex acid into its components was, however, accomplished by treating its acetone solution with hydrochloric acid. The acetonyl compound of the ester separated, and from the mother liquor the component acid was easily obtained. This acid was shown to be a monohydroxy acid with the formula $C_{32}H_{50}O_5$. The methoxyl

determination showed the ester group originally present in hederagenin methyl ester to be intact. This substance is, therefore, the monomethyl ester of a dibasic monohydroxy acid, *hederagic acid*. From this ester the dimethyl ester was readily prepared by means of methyl sulfate, and in turn gave a monoacyl compound. The carboxyl group in hederagic acid must have been formed by the oxidation of a terminal primary alcoholic group, CH_2OH .

The above results permit the conclusion that hederagenin is a dihydroxy acid possessing a primary and a secondary alcoholic group which form a substituted trimethylene glycol. This relationship is shown as follows:



If these conclusions are correct the above acid oxidation product must be a β -hydroxy acid. Further work is in progress to substantiate this conclusion.

EXPERIMENTAL.

Sulfite of Hederagenin Chloride.—5 gm. of hederagenin were warmed on the bath in 10 cc. of thionyl chloride for 30 minutes. Solution occurred rapidly. On diluting the reaction mixture with ligroin a pap of colorless needles separated, which were collected with ligroin. The mother liquor from the 3 gm. which were obtained yielded on concentration *in vacuo* an additional 1.5 gm. Recrystallized from ligroin it formed long broad colorless needles which melted after preliminary sintering at $251\text{--}253^\circ\text{C}$. with effervescence. The chloride is easily soluble in benzene and less readily in ether and acetone.

$\text{C}_{21}\text{H}_{47}\text{O}_4\text{S}\cdot\text{Cl}$.	Calculated.	C 67.52, H 8.60, Cl 6.44.
	Found (a).	" 67.51, " 8.53.
	(b).	" 67.21, " 8.40.
	(c).	" 6.40.
	(d).	" 6.49.

Sulfite of Hederagenin Methyl Ester.—When the chloride was boiled with an excess of methyl alcohol it was rapidly replaced by

the sparingly soluble ester with the liberation of hydrochloric acid. The heating was continued for several hours. Recrystallized from a necessarily large volume of methyl alcohol the ester formed delicate needles which melted at 235–237°. On recrystallization from ligroin this was raised to 238–239°. Although this melting point was practically the same as that given by hederagenin methyl ester the sulfite was much more sparingly soluble than the latter and a mixture of the two substances melted below 200°. The sulfite is readily soluble in chloroform, benzene, acetone, and ether. It disclosed its nature also by saponification.

0.1138 gm. was refluxed in 15 cc. of absolute alcohol and 15 cc. of 0.1 N NaOH for 2 hours and was then titrated against phenolphthalein. Calculated, 4.15 cc. Found, 4.60 cc. A substance separated from the saponification mixture which proved to be hederagenin methyl ester and on acidification sulfur dioxide was readily detected.

$C_{32}H_{50}O_6S$. Calculated. C 70.27, H 9.22.
Found. " 70.50, " 9.26.

Sulfite of Hederagenin Amide.—A solution of the chloride in chloroform was shaken with an excess of concentrated ammonia. After washing the chloroform solution with water and drying over Na_2SO_4 , the chloroform was removed. The residue when dissolved in 95 per cent alcohol gradually crystallized. When recrystallized from alcohol it formed aggregates of leaflets which as stated by van der Haar contained 1 mol of H_2O and melted at 285°.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$C_{31}H_{49}O_4NS \cdot H_2O$. Calculated. H_2O 3.27.
Found. " 4.09.

Anhydrous Substance.

$C_{31}H_{49}O_4NS$. Calculated. C 69.99, H 9.29.
Found (a). " 69.68, " 9.08.
(b). " 70.01, " 9.07.

0.1235 gm. of substance was refluxed with 15 cc. of 0.1 N NaOH and 15 cc. of alcohol and titrated back against phenolphthalein. Calculated for SO , 4.63 cc. Found, 4.66 cc. When the saponification mixture was acidified, SO_2 was easily detected.

Hederagenin Amide.—The sulfite was saponified by boiling with

an excess of sodium hydroxide in alcoholic solution for 1 hour. The mixture was carefully diluted with water, causing the separation of a flocculent precipitate of delicate needles. The collected substance was recrystallized from 95 per cent alcohol. It formed needles which contained 3 mols of water. The substance melted at 300–303° and was rather sparingly soluble in the usual solvents.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$\text{C}_{31}\text{H}_{51}\text{O}_3\text{N} \cdot 3\text{H}_2\text{O}$. Calculated. H_2O 10.02.

Found. " 9.56.

Anhydrous Substance.

$\text{C}_{31}\text{H}_{51}\text{O}_3\text{N}$. Calculated. C 76.64, H 10.59.

Found. " 76.59, " 10.43.

Acetonyl Hederagenin Methyl Ester.—2 gm. of the methyl ester were dissolved in 40 cc. of dry acetone. On standing, the mixture remained clear, but if a few drops of concentrated hydrochloric acid were added, a mass of glistening leaflets at once deposited. Recrystallized from absolute alcohol, the very sparingly soluble acetonyl compound formed lustrous thin long platelets which melted at 250–252°. It is easily soluble in chloroform and benzene and appreciably soluble in ether and acetone. When dissolved in H_2SO_4 and warmed, it develops at first a yellow color which changes through orange to a fluorescent orange-red. When boiled in alcohol to which concentrated hydrochloric acid had been added it dissolved, owing to hydrolysis of the acetonyl group, and hederagenin methyl ester was readily recovered from the solution. After boiling with acetic anhydride, it dissolved but separated unchanged on cooling. This is convincing evidence that the original hydroxyl groups of hederagenin had been covered.

$\text{C}_{35}\text{H}_{55}\text{O}_4$. Calculated. C 77.71, H 10.44.

Found. " 77.59, " 10.11.

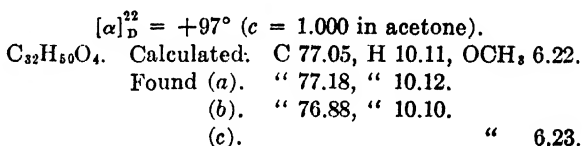
Hederagenin methyl ester prepared from ivy leaves when submitted to the above reaction yielded the same acetonyl compound as shown by analysis, melting point, and other properties.

Found. C 77.56, H 10.10.

Oxidation of Hederagenin Methyl Ester.

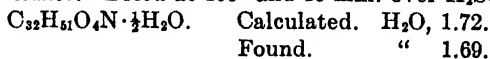
Hederagonic Methyl Ester.—52 gm. of the methyl ester were dissolved in 800 cc. of dry acetone and shaken with 26 gm. of

finely powdered potassium permanganate. After a few minutes the mixture warmed appreciably and within 10 to 15 minutes the permanganate was used up. After cooling the MnO_2 was filtered and washed well with dry acetone. The filtrate, colored deeply by colloidal MnO_2 , was concentrated to a syrup which rapidly crystallized. The residue was boiled with sufficient methyl alcohol to dissolve all but the remaining MnO_2 which was then collected with bone-black. As the ketone separated only incompletely from methyl alcohol the filtrate was concentrated until crystallization began. 19 gm. of the crude ketone were obtained. Recrystallized from methyl alcohol in which it is rather sparingly soluble, it formed aggregates of stout plates and prisms which melted slowly at $217\text{--}218^\circ$ with preliminary sintering. It is easily soluble in chloroform, benzene, ether, and acetone, and is appreciably soluble in hot ligroin. In H_2SO_4 it dissolves with a straw color which changes, on warming, to a fluorescent orange-red. If H_2SO_4 is added to a solution in chloroform containing a few drops of acetic anhydride a deep purple color develops between the layers. It does not yield an acetonyl compound.

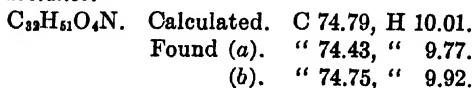


Hederagonic Methyl Ester Oxime.—This was prepared from the ester in methyl alcoholic solution with hydroxylamine hydrochloride and sodium acetate. Recrystallized from methyl alcohol it formed colorless microscopic platelets which melted and effervesced at $211\text{--}213^\circ$ after preliminary sintering.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .



Anhydrous Substance.



Hederagonic Methyl Ester-o-Bromobenzoate.—The ketone ester was acylated in pyridine solution with *o*-bromobenzoyl chloride.

After considerable difficulty the crude amorphous substance was made to crystallize from alcohol. Recrystallized from this solvent in which it is very sparingly soluble it separated as colorless prisms or needles which melted at 236–238° after slight preliminary softening.

$C_{33}H_{52}O_5Br$. Calculated. C 68.68, H 7.84, Br 11.73.
Found. " 68.60, " 7.74, " 11.69.

Hederagenin Methyl Ester Hederagic Monomethyl Ester.—

The washed MnO_2 residue obtained from the oxidation mixture was boiled with 4 liters of 70 per cent alcohol until thoroughly disintegrated. The filtrate was concentrated to dryness on the water bath. The residue was digested with a small volume of hot 95 per cent alcohol during which copious separation of a potassium salt occurred. After cooling and standing the thick pap of needles was collected with alcohol. 9.5 gm. were obtained. The mother liquor contained K_2CO_3 and other oxidation products which are under investigation. The potassium salt was dissolved in sufficient hot 50 per cent alcohol and the solution was then acidified with acetic acid. The acid separated quickly as delicate needles which were recrystallized from dilute alcohol. It softened from 242° to a paste which melted from 249 to 255°.

On investigation this acid proved to be a sort of molecular compound of hederagenin methyl ester with the simpler acid to be described below.

$[\alpha]_D^{25} = +84.6^\circ$ ($c = 1.04$ in 95 per cent alcohol).

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$C_{32}H_{52}O_4 \cdot C_{32}H_{50}O_5 \cdot 2H_2O$. Calculated. H_2O 3.43.
Found. " 3.73.

Anhydrous Substance.

$C_{32}H_{52}O_4 \cdot C_{32}H_{50}O_5$. Calculated. C 75.68, H 10.13.
Found (a). " 75.59, " 9.89.
(b). " 75.22, " 9.88.

Although the properties of hederagenin methyl ester itself and its inability to form stable salts seemed to preclude the possibility that it was carried along mechanically with the acid to be described below; nevertheless, the following attempt was made to cause a separation if the substance were really a mixture.

The above acid was dissolved in about 20 parts of hot 95 per

cent alcohol. A drop of phenolphthalein was added and, while hot, this was followed by just sufficient alcoholic potassium hydroxide to produce the color change. A salt rapidly separated and, because of the neutral character of the ester, if uncombined, it should have remained in solution. However, the salt proved to be the salt of the unchanged complex acid. It was dissolved in hot 50 per cent alcohol, and the mixture acidified with acetic acid. The collected acid was dissolved in hot alcohol and carefully treated with water. It separated again as delicate needles which melted at 252–255° to a turbid liquid which cleared at 257°.

0.1071 gm. dissolved in absolute alcohol when titrated against phenolphthalein required 1.25 cc. of 0.1 N NaOH. Calculated, 1.05 cc.

$$[\alpha]_D^{22} = +84^\circ \text{ (} c = 1.025 \text{ in 95 per cent alcohol).}$$

Air-Dry Substance. Dried at 100° and 15 mm. over H₂SO₄.

C₃₂H₆₂O₄ · C₃₂H₆₀O₆ · 3H₂O. Calculated. H₂O 5.04.

Found. " 4.95.

Anhydrous Substance.

C₃₂H₆₂O₄ · C₃₂H₆₀O₆. Calculated. C 75.68, H 10.13.

Found. " 75.37, " 9.79.

The acid does not react with ketone reagents and in an attempt to prepare a bromobenzoate by the pyridine method the dibromobenzoate of hederagenin methyl ester was obtained. Cleavage of the acid into its components was readily accomplished in the following manner.

Hederagic Monomethyl Ester.—5 gm. of the double compound were dissolved in 70 cc. of acetone. The solution remained clear, but when 1 cc. of HCl (1.19) was added leaflets immediately appeared which proved to be the acetyl compound of hederagenin methyl ester. The yield was 2.8 gm. On careful dilution of the mother liquor the component acid crystallized as delicate needles which were collected with 50 per cent alcohol. For recrystallization this was suspended in sufficient 50 per cent alcohol, heated, and then dissolved by the careful addition of ammonia. An excess was avoided, owing to the sparing solubility of the ammonium salt. The rapidly filtered solution was treated while hot with dilute HCl. Recrystallized again from dilute alcohol it formed needles which melted at 270–272° after preliminary sintering. Although this substance gave satisfactory

analytical figures an additional purification was effected over the *sodium salt*. This was prepared by treating the alcoholic solution of the acid with sodium acetate. The sparingly soluble sodium salt which gradually separated was recrystallized from alcohol and then reconverted into the acid. The latter now melted at 274–276° with slight preliminary sintering. It dissolves in hot methyl alcohol and crystallizes again as broad 4-sided, often rhombic plates. If the cold dilute alcoholic solution of the salt is acidified it forms a gel which slowly crystallizes. In H_2SO_4 it gives at first a colorless solution which changes on warming to a fluorescent orange-red. The acid did not form an oxime. In acylation experiments, although a reaction was apparent, attempts to isolate the reaction products in crystalline form were unsuccessful.

$$[\alpha]_D^{20} = +89.5^\circ \text{ (} c = 1.005 \text{ in 95 per cent alcohol).}$$

Titration established the presence of one COOH group. 0.2349 gm. of anhydrous substance was titrated in alcoholic solution with 0.1 N NaOH against phenolphthalein. Calculated, 4.55 cc. Found, 4.25 cc.

Air-Dry Substance. Dried at 100° and 15 mm. over H_2SO_4 .

$\text{C}_{32}\text{H}_{40}\text{O}_5 \cdot \text{H}_2\text{O}$. Calculated. H_2O 3.38.

Found. " 2.56.

Anhydrous Substance.

$\text{C}_{32}\text{H}_{40}\text{O}_5$. Calculated. C 74.65, H 9.80, OCH_3 6.03.

Found (a). " 74.42, " 9.61.

(b). " 74.67, " 9.62.

(c). " 6.41.

Hederagic Dimethyl Ester.—This was prepared from the acid in methyl alcoholic solution with methyl sulfate and KOH. The reaction mixture was extracted with ether and the latter was purified by extraction with dilute alcoholic alkali. Alcohol was necessary to prevent the formation of an emulsion. The ester separated from alcohol as platelets which melted at 244–246°.

$\text{C}_{33}\text{H}_{42}\text{O}_5$. Calculated. C 74.94, H 9.92.

Found. " 74.95, " 9.83.

Hederagic Dimethyl Ester-o-Bromobenzoate.—The ester was acylated as usual in pyridine solution with the acid chloride. Recrys-

tallized from methyl alcohol it slowly separated as sparingly soluble flat needles and leaflets which melted at 194–197° with preliminary sintering.

$C_{40}H_{60}O_6Br$. Calculated. C 67.48, H 7.79, Br 11.24.
Found. " 67.53, " 7.69, " 11.30.

THE ACTION OF TRYPSIN ON INSULIN.

By D. A. SCOTT.

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(Received for publication, October 22, 1924.)

Various investigators have obtained very different yields of insulin from the pancreas. Reference to the important contributions in this field may be found in a recent article from this laboratory (1). The great variation in the yields of insulin may be partly due to different methods of assay, nevertheless, it is obvious that there are factors in the preparation as yet not understood. It was thought that a more complete study of the action of trypsin on insulin might help to explain the discrepancies in the yields.

Banting and Best (2) were the first to observe that trypsin destroys insulin. Dudley (3) and later Witzemann and Livshis (4) showed that both pepsin and trypsin destroy insulin. Shonle and Waldo (5) confirmed the findings of the previous investigators. Epstein and Rosenthal (6) in more recent work state that the action of trypsin on insulin is immediate and that the insulin may be recovered by increasing the acidity of the solution. They also state that if trypsin is injected directly after the administration of insulin the action of the latter is inhibited. They conclude that the action of the trypsin is not proteoclastic.

EXPERIMENTAL.

It was first necessary to determine approximately the least amount of trypsin necessary to inactivate completely a known amount of insulin. This was done by adding various amounts of a solution of trypsin to 10 units of insulin (1 cc., pH 2.5), and immediately injecting the mixture into a series of standard test rabbits. In Table I, I have indicated the manner in which the amount of trypsin was roughly determined.

Table I shows that 1 mg. of trypsin per 10 units of insulin is not

sufficient to inactivate immediately the insulin. Therefore, in all subsequent work 10 mg. of trypsin per 10 units of insulin were used. The trypsin¹ was made up in water so that 1 cc. contained 10 mg.

Attempts to Recover Latent Potency.

Attempts to recover the potency from the insulin-trypsin complex were made by: (1) varying the acidity, (2) varying the temperature, (3) varying the solvent, and (4) the use of detergents (sodium taurocholate, sodium glycocholate, saponin, and benzoates).

The first series of experiments was attempts to recover by acid hydrolysis the potency from insulin inactivated by trypsin. Trypsin was added to the insulin solution in such amounts that

TABLE I.

Insulin.	Trypsin.	Remarks
<i>units</i>	<i>mg.</i>	
10	0.1	Convulsions in all rabbits.
10	1	3 units (average figure).
10	10	No significant change in blood sugar.
10	100	Hyperglycemia in all rabbits.

1 mg. of trypsin was present for every unit of insulin. The mixture was then hydrolyzed with HCl, added in sufficient quantities to give an acidity in the first experiments of pH 2.5. In these experiments the temperature and time were variables, while the acidity was constant (pH 2.5). After hydrolysis, the equivalent of 10 units was administered at each injection into standard test rabbits and the amount of reclaimed activity determined. The results are expressed in Charts 1 to 3. Some of the numerical values obtained are indicated on the charts.

Chart 1 shows that little potency is recovered by heating under 60° C. and that the recovered activity is rapidly lost at or above 110° C. (pH 2.5). It also indicates that the rate of disappearance of the recovered potency is roughly proportional to the amount reclaimed at any time. This is interesting in the light of the rapid

¹ Digestive Ferments Company trypsin was used.

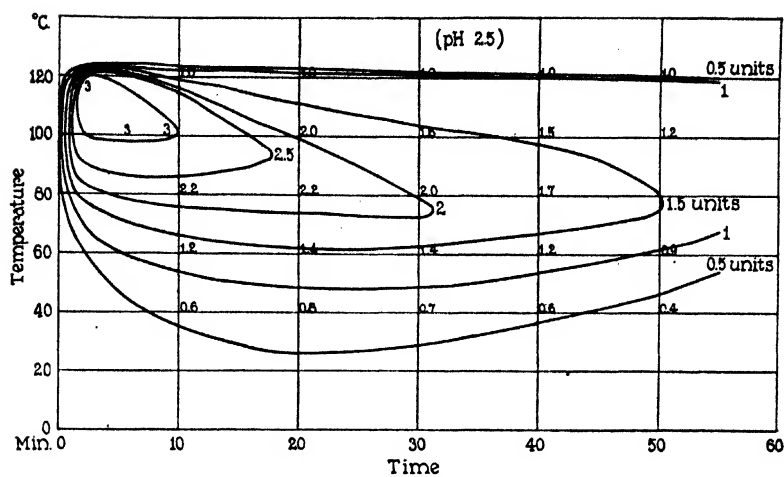


CHART 1.

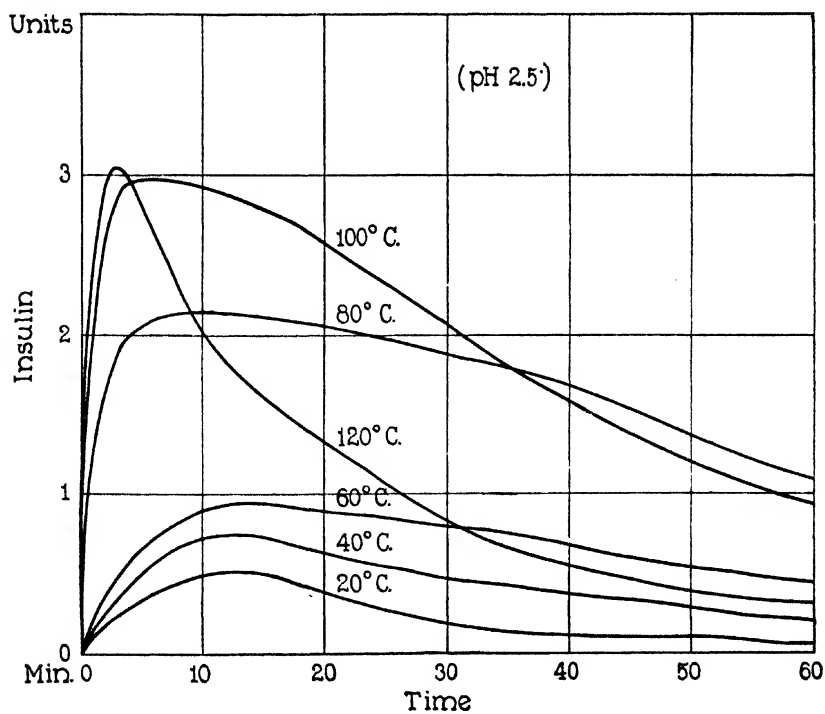


CHART 2.

disappearance of potency when very high yields of insulin are obtained from the pancreas.

In Chart 2 units of regained potency are plotted against time of heating at different temperatures. This chart shows that the initial rate of recovery increases directly with the temperature. It would also appear that the time of heating at any temperature to secure maximum activation is roughly proportional to the temperature. The best procedure for the recovery of potency at this acidity is heating the solution for 3 minutes at a temperature of approximately 110°C . In practice, however, it is inconvenient to heat for such a short period. For this reason, in a second series of

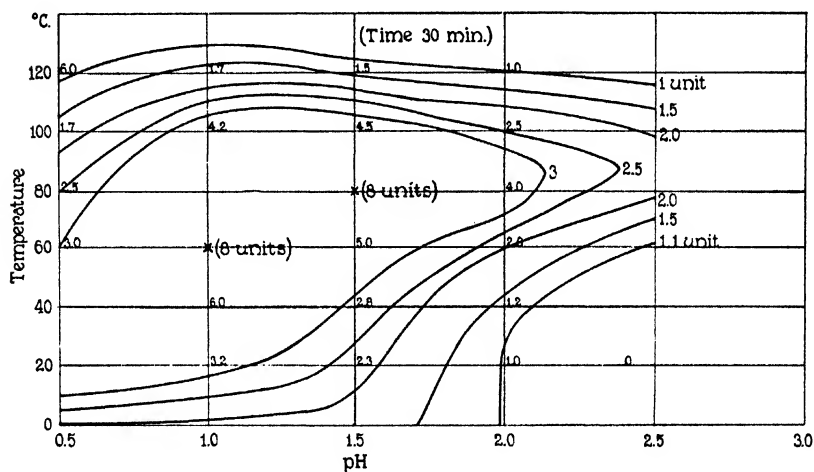


CHART 3.

experiments, the time of heating was set at 30 minutes and the temperature and acidity varied. The results are illustrated in Chart 3.

Chart 3 shows that over a considerable range for each pH value there is an optimum temperature for the activation of the latent insulin. When the temperature is increased and the acidity decreased very little activity is recovered. However, when the acidity is increased and the temperature decreased, as far as we have investigated, the activity continues to be recovered in large amounts. Further research in this direction was prevented by the high salt content formed on neutralizing the acid before the

injection. A 30 minute hydrolysis at pH 1.5 at 80° C. was used in all subsequent work as it offered a fair compromise between efficiency in recovery of activity and ease of control. These conditions permitted a recovery of 80 per cent of the initial potency.

Sulfuric acid was found to be as effective as hydrochloric acid for similar pH values. The detergents, sodium taurocholate, sodium glycocholate, and saponin, were completely ineffective in recovering the latent potency.

A large part of the insulin could be recovered from the insulin-trypsin complex by acid alcohol as is shown in the following experiment. To 20 cc. of 10 unit insulin were added 20 cc. of trypsin (200 mg.). The mixture was shaken and the acidity lowered to pH 1.5. Immediately 4 volumes of 95 per cent alcohol were added. The mixture was left to extract for 2 hours at room temperature. The precipitate which formed was filtered off and the alcohol removed *in vacuo*. The concentrate was made up to its original volume and assayed for potency. Approximately 60 per cent of the insulin was recovered.

Treatment of the inactive insulin with sodium benzoate was also effective in recovering part of the latent potency. 2 gm. of sodium benzoate were added to 20 cc. of insulin, made inactive with trypsin. After standing 4 hours, the solution was acidified with hydrochloric acid and the benzoic acid ethered out. Approximately half of the original potency was recovered.

Benzoic acid alcohol was very effective in recovering the latent potency. 20 cc. of trypsin solution were added to 20 cc. of insulin. This mixture was shaken and immediately 40 cc. of a saturated solution of benzoic acid alcohol were added. The mixture was acidified with 1 cc. of 1 N HCl. After standing 15 minutes the mixture was heated in a boiling water bath until most of the alcohol distilled off. Ether was then added to remove the benzoic acid. The aqueous fraction, after the removal of traces of ether *in vacuo*, contained practically all of the original potency.

Adsorption Phenomenon.

In the following experiments the action of inactivated trypsin on insulin was studied. 20 cc. of a solution of trypsin (200 mg., pH 2.5) were killed by heating at 80°C. for 30 minutes. After

cooling, this solution was added to 20 cc. of 10 unit insulin. No loss in potency occurred. In a second experiment 50 cc. of the insulin were inactivated with trypsin in the usual manner. After

TABLE II.

Amount injected.	Experiment.	Blood sugar.*		Amount reclaimed.
		Before.	After (1½ hrs.).	
cc.				per cent
0.6	20 cc. insulin + 20 cc. trypsin inactivated by heat.	0.104	0.045	100
0.8	20 cc. insulin + 20 cc. trypsin heated together.	0.110	0.048	80
0.8	After standing 1 week.	0.098	0.040	80

* All estimations of potency of insulin solutions were made by injecting graded doses into a series of standard rabbits. The blood sugar of each rabbit was determined 1½, 3, and 5 hours after the injection. The potency was calculated as described in a recent article from the Insulin Committee's laboratory by Macleod and Orr (7). As it would require too much space to report all the blood sugar values, only the 1½ hour blood sugar in one typical experiment is recorded.

TABLE III.

Amount injected.	Experiment.	Blood sugar.		Amount re-claimed.
		Before.	After (1½ hrs.).	
cc.				per cent
0.3	50 cc. supernatant liquid from charcoal.	0.104	0.110	10
2	50 " " " " "	0.110	0.066	
4	50 " " " " "	0.118	0.040	
0.3	50 cc. suspension of charcoal.	0.110	0.104	0.0
1	50 " " " " "	0.098	0.110	
4	50 " " " " "	0.110	0.116	
0.3	45 cc. product reclaimed from charcoal by benzoic acid alcohol.	0.110	0.066	60
0.6	" "	0.124	0.040	

the acidity had been adjusted to pH 1.5 the solution was heated at 80°C. for 30 minutes. On cooling, the acidity was adjusted to pH 2.5 and no inactivation of insulin occurred even on standing 1 week at room temperature (Table II).

Since charcoal readily adsorbs insulin from an aqueous solution, we were interested to determine the activity of the insulin-charcoal complex. 0.5 gm. of powdered charcoal was shaken for 2 minutes with 50 cc. of 10 unit insulin at pH 2.5. The charcoal was removed by centrifuging, washed with water, and the washings were added to the supernatant liquid. An assay showed that there was a 90 per cent loss of potency. The charcoal was shaken with 50 cc. of water and portions of the suspension equivalent to 3, 10, and 40 units of insulin were injected. In each case the blood sugar remained normal. The remaining charcoal was centrifuged off and the insulin extracted with benzoic acid alcohol (8). Approximately 60 per cent of the original potency was recovered (Table III).

Proteoclastic Action.

The proteoclastic action of trypsin on insulin was next investigated. 25 cc. of 10 unit insulin, pH 2.5, were mixed with 25 cc. of trypsin solution (250 mg.). After adjusting to pH 7.5 and adding a few drops of xylene the solution was incubated at 40°C. Samples were withdrawn hourly and attempts were made to recover the latent potency by heating at 80°C. for 30 minutes at pH 1.5. The latent potency of the original solution fell progressively to half its initial value in 4 hours. After standing overnight no activity could be recovered by acid hydrolysis or by acid alcohol extraction, even on injecting quantities equivalent to 3, 10, and 60 units of the original material. Control experiments on insulin alone under similar conditions showed little loss in activity. However, when the insulin-trypsin solution (pH 7.5) was left in the ice chest overnight, most of the activity could be reclaimed (Table IV). In some experiments strict aseptic technique was employed.

Action in Vivo.

The action of trypsin on insulin *in vivo* was studied. Standard test rabbits were injected with 3 units of insulin and immediately afterwards with 0.3 cc. of trypsin (3 mg.). The blood sugar determinations showed that there was no appreciable loss in potency. The same results were secured when 0.6 and 0.9 cc. of the trypsin solution were injected. Thus more than three times the quantity

TABLE IV.

Amount injected.	Experiment.	Blood sugar.		Amount re-claimed.
		Before.	After (1½ hrs.).	
cc.				per cent
1	25 cc. insulin + 25 cc. trypsin (pH 7.5).	0.110	0.098	0.0
4	25 " " + 25 " " (" 7.5).	0.098	0.104	
0.5	Portions of solution after 4 hrs. incubation hydrolyzed at 80°C. (pH 1.5).	0.118	0.072	40
1.5	" "	0.110	0.048	
0.3	Tryptic digest after 12 hrs. hydrolyzed at 80°C. (pH 1.5).	0.104	0.110	0.0
1.0	" "	0.098	0.098	
6.0	" "	0.098	0.110	
0.6	20 cc. insulin + 20 cc. H ₂ O, pH 7.5 at 40°C. for 12 hrs.	0.118	0.060	90
0.8	" "	0.110	0.048	
0.6	25 cc. insulin + 25 cc. trypsin, pH 7.5 in ice chest 12 hrs., activated by acid.	0.110	0.066	70
0.8	" "	0.098	0.048	

TABLE V.

Amount injected.	Experiment.	Blood sugar.		Amount re-claimed.
		Before.	After (1½ hrs.).	
cc.				per cent
0.3	10 unit insulin immediately followed by 0.3 cc. of trypsin (subcutaneous).	0.098	0.040	90
0.3	10 unit insulin immediately followed by 0.6 cc. of trypsin (subcutaneous).	0.110	0.054	90
0.3	10 unit insulin immediately followed by 0.9 cc. of trypsin (subcutaneous).	0.104	0.048	80
0.3	10 unit insulin immediately followed by 0.3 cc. of trypsin (intravenous).	0.098	0.040	100

of trypsin necessary to inactivate completely insulin *in vitro* had no appreciable effect *in vivo*. Both subcutaneous and intravenous injections were made (Table V). Injections of larger amounts of trypsin produce hyperglycemia which counteracts the effect of insulin.

DISCUSSION.

It has been shown, confirming Epstein and Rosenthal, that insulin is immediately inactivated by trypsin *in vitro* at certain acidities. Various methods of recovering the insulin from the trypsin-insulin complex have been tried. By immediately heating the complex to 80°C. for 30 minutes (pH 1.5) 80 per cent of the original potency is recovered. Acid alcohol or sodium benzoate is also effective in recovering a large part of the insulin made inactive by trypsin. Since acid alcohol has been found to be very effective in extracting insulin from the pancreas it is possible that much of the insulin exists there in an inactive form. Other results which indicate that insulin may exist in the body in an inactive form are: (1) A simple aqueous extraction of the pancreas thus far has resulted in low yields of insulin. (2) Insulin is present in appreciable amounts in diabetic tissues. (3) The best yields of insulin from urine have been obtained by the benzoic acid method of purification. These yields may be due to the liberation of the latent potency by the strong hydrochloric or benzoic acid. (4) On several occasions the potency of the final product has been increased several fold by the use of strong hydrochloric acid.

The preliminary action of trypsin on insulin would seem to be similar to the action of charcoal on insulin, both since the complex is not active and since the activity may be recovered by suitable extraction.

The results of the experiments on the proteoclastic action of trypsin on insulin would suggest that the primary action is a mutual adsorption phenomenon and that this is followed by complete proteoclastic destruction. This is in accord with the theory of enzyme action (9).

The amount of trypsin necessary to inactivate completely insulin *in vitro* had no appreciable effect *in vivo* when injected immediately after the insulin. These results are contrary to

those of Epstein and Rosenthal who found that trypsin inactivates insulin *in vivo* as well as *in vitro*. Probably their results may be attributed to an overdose of insulin, which may produce surprisingly little effect on the blood sugar, or to an overdose of trypsin which produces hyperglycemia.

The experiments on the recovery of the latent potency and the proteoclastic action of trypsin on insulin indicate the advisability of using fresh pancreas and high acidity for obtaining a high yield of insulin.

CONCLUSIONS.

1. Insulin is immediately inactivated by trypsin at certain acidities.

2. The latent potency may be partly reclaimed by suitably adjusting the temperature and acidity, or by the use of alcohol or benzoates.

3. When insulin and trypsin are incubated together (pH. 7.5, 40°C.) for about 12 hours no insulin can be recovered by the procedures referred to above.

4. Trypsin in amounts which inactivate insulin *in vitro* does not inhibit insulin when the substances are injected separately.

I wish to acknowledge by indebtedness to Mr. K. L. MacAlpine for his efficient assistance. I also wish to thank Dr. C. H. Best and Dr. A. Hunter for their helpful criticism.

Addendum.—A paper by Epstein and Rosenthal on the nature of the action of trypsin on insulin (10) has appeared since our communication was sent to press. These writers were able to activate the insulin after a 42 hour digestion by trypsin. I have made numerous attempts to repeat these experiments. Purified trypsin was used. These attempts have been unsuccessful. Even benzoic acid alcohol, which in my experience is by far the most efficient agent for the recovery of latent potency, has given negative results in these experiments.

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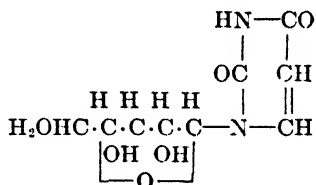
PHENYLHYDRAZINO DERIVATIVES OF PYRIMIDINES.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 27, 1925.)

The object of this communication is to present some additional indirect evidence in support of the theory regarding the structure of pyrimidine ribosides expressed in a previous publication. Theoretical considerations were there developed which lead to the conclusion that in uridine the union between ribose and pyrimidine is in position (3) of the latter, thus giving for uridine the graphic expression.



It was attempted to substantiate further the theory by methylation of the nucleoside as was done in the case of xanthosine. Work in this direction has not as yet yielded satisfactory results. Thus direct experimental evidence in support of the theory is still lacking. On the other hand, the mass of indirect evidence has increased. The phenylhydrazine derivatives to be discussed here furnish substantial evidence of this type. However, before discussing these, it may be of service to recall some qualitative tests which also support the theory given in the above graphic expression of uridine.

At a time when the details of the structure of nucleic acids were very little known, Burian compared the action of diazobenzenesulfonic acid on nucleic acids with that on some substituted purines. He found that this reagent formed a dye with non-substituted purines and with 1,3-disubstituted purines, but

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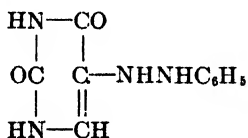
gave no color reaction with purines substituted in position (7), and likewise it gave none with nucleic acids. Burian suggested that in nucleic acids the purines are attached to the rest of the nucleic acid through the nitrogen atom in position (7). Hans Fischer later pointed out that the substitution in position (8) of a purine also interfered with the formation of the dye. The observations of Burian on purines induced other workers on pyrimidine derivatives to study the reaction between these substances and diazobenzenesulfonic acid. Evans, Steudel, Pauli, and Burian reported casual observations, but T. B. Johnson and S. H. Clapp made a systematic study of the influence of substitution in pyrimidines on the formation of a dye with diazobenzenesulfonic acid. The conclusion from their observations was that pyrimidines substituted in position (3) gave a negative test with this reagent, and that substitution in any other position did not interfere with the reaction. Following the example of Burian, they suggested that the pyrimidine bases are linked to the rest of the nucleic acid molecule through the nitrogen atom in position (3).

Another color test for uracil and cytosine was introduced by H. L. Wheeler and T. B. Johnson. They found that a purple precipitate formed when baryta water was added to a solution of uracil or cytosine treated with 2 equivalents of bromine. To the knowledge of the writer this test has not been studied systematically on substituted uracils. In the original paper a negative result was reported with thymine and H. L. Wheeler and L. M. Liddle report a negative test with uracil-3-acetic acid. We have tested the reaction on 1,3-dimethyluracil and obtained a negative result. Addition of barium hydroxide subsequent to treatment with bromine gave rise to a perfectly white precipitate. 3-monomethyluracil reacted identically. Thus, uracils, substituted in position (3), seem to be characterized by their inability to react with diazobenzenesulfonic acid and also by a negative Wheeler and Johnson color test.¹

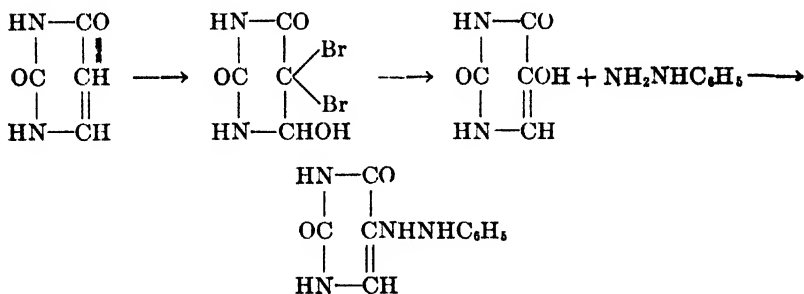
¹ Wheeler and Johnson later reported a positive test with 3-methyluracil, and Behrend and von Loeben and Behrend and Prusse report positive tests with 1-methyl- and with 3-methylisodialuric acids. The purity of the samples used in the present experiments was tested by analysis, and by melting points. Furthermore, these substances reacted as expected with benzenesulfonic acids.

Uridine had not been tested previously in regard to these two reagents. It was now found that uridine responded negatively to these two color tests, thus exhibiting the behavior of 3-substituted uracils.

Of greater interest than the color tests is the reaction of uracil with phenylhydrazine. When uracil is treated with 2 equivalents of bromine and subsequently with phenylhydrazine, a substance of the following composition is obtained.

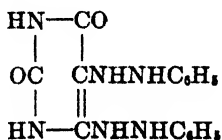


5-bromouracil treated in the same way with phenylhydrazine fails to give an insoluble derivative; on the other hand, isobarbituric acid gives the same derivative directly on treatment with phenylhydrazine without previous treatment with bromine. 5-bromouracil treated first with an excess of bromine and then with phenylhydrazine gives the same phenylhydrazine derivative. Thus the mechanism of the reaction is undoubtedly the following.



4-methyluracil and 5-methyluracil, under identical treatment, do not give an insoluble product.

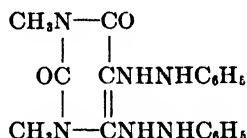
Isodialuric Acid.—When this acid is treated with phenylhydrazine, it forms the following derivative.



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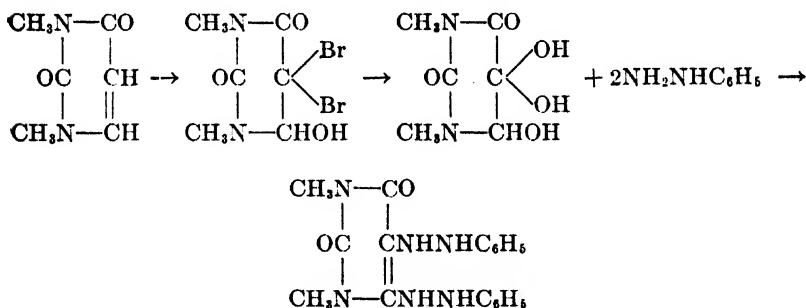
Alloxan treated in the same way did not form a phenylhydrazine derivative.

1,3-Dimethyluracil.—When this substance is treated in the identical way as the non-substituted uracil, a 4,5-disubstituted product is formed of the composition



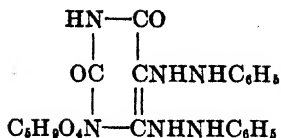
This derivative differs markedly from that of isodialuric acid. It has a deep orange color and the appearance of a sugar osazone.

Thus apparently the substitution in position (3) causes a different course of the reaction; namely, the following.

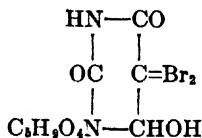


Thus when uracil is reacted upon under the above conditions the precursor of the hydrazide is isobarbituric acid and when 3-substituted uracil is the starting material, the precursor is isodialuric acid. These hydrazino derivatives of isobarbituric and isodialuric acids form so readily and have such definite melting points that they may serve for purposes of identification.

Uridine.—A derivative of phenylhydrazine with uridine has been previously described by Levene and La Forge. At the time, however, there was only a limited quantity of the material on hand, and the purification of the substance was not carried far enough. However, when sufficiently recrystallized, the substance analyzed for a dihydrazino derivative of the following composition.



The substance has the typical appearance of an osazone and resembles very much the derivative of 1,3-dimethyluracil. The same substance is obtained when the intermediate derivative



is treated with lead hydroxide prior to treatment with phenylhydrazine.

5-bromouridine, on the other hand, like 5-bromouracil, does not react readily with phenylhydrazine. However, if it is treated with bromine water prior to digesting with phenylhydrazine, the derivative is formed.

Thus, the mechanism of the reaction is similar to that which takes place with 1,3-dimethyluracil. Since uridine is not substituted in position (1), the formation of the dihydrazine derivative of uridine furnishes additional evidence in favor of the structure expressed above.

EXPERIMENTAL PART.

The same procedure was followed for the preparation of all the dihydrazino derivatives. 1.0 gm. of the substance was dissolved or suspended in water depending upon its solubility. Bromine was added until the color of the solution remained permanently yellow. The excess of bromine was then removed by a current of air. To the solution 3.0 gm. of sodium acetate and 5.0 gm. of phenylhydrazine dissolved in glacial acetic acid were added and the solution was allowed to stand on the steam bath. In some instances, a crystalline substance begins to settle out after a few minutes; in other instances, as in the case of 1,3-dimethyluracil, the substance makes its appearance gradually. In the case of uridine, the reaction seems complete in from 1 to 1½ hours.

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For other substances it is advisable to allow the solution to remain on the steam bath for several hours.

5-Phenylhydrazinouracil from Uracil.—This substance had a light grayish yellow color, and consisted of microscopic elongated prisms. It was very insoluble in both hot and cold water and very insoluble in neutral organic solvents. It is somewhat soluble in boiling glacial acetic acid. For purification it is sufficient to extract the precipitate several times with cold methyl alcohol. The substance melted at 252°C. and had the following composition.

0.0988 gm. substance: 0.1998 gm. CO₂ and 0.0432 gm. H₂O.

0.0863 " " : 18.80 cc. nitrogen gas at $t^{\circ} = 22^{\circ}\text{C.}$ and $p = 772$ mm.

C ₁₀ H ₁₀ N ₄ O ₂ .	Calculated.	C 55.02, H 4.62, N 25.69.
	Found.	" 55.14, " 4.89, " 25.61.

5-Phenylhydrazinouracil from Isobarbituric Acid.—This is prepared directly by adding to a solution of 1.0 gm. of the substance 3 gm. of phenylhydrazine in glacial acetic acid solution. The light yellow (somewhat grayish) precipitate, consisting of small prismatic needles, appears in about 10 minutes. The substance was also obtained from a solution containing only 0.020 gm. of the substance. It had all the properties of the substance obtained from uracil and analyzed as follows:

0.1004 gm. substance: 0.2196 gm. CO₂ and 0.0430 gm. H₂O.

0.0958 " " : 21.70 cc. nitrogen gas at $t^{\circ} = 23^{\circ}\text{C.}$ and $p = 755.9$ mm.

C ₁₆ H ₁₇ N ₅ O ₂ .	Calculated.	C 59.04, H 5.26, N 25.84.
	Found.	" 59.64, " 4.86, " 26.11.

The substance should have been further purified, but lack of material prevented it. There is, however, no doubt as to its composition.

1,3-Dimethyl, 4,5-Diphenylhydrazinouracil.—Only 0.5000 gm. of the substance was used for the preparation of this derivative. The substance settled out as a bright orange crystalline deposit having the appearance, under the microscope, of a glucosazone. The substance was repeatedly extracted with methyl alcohol. It melted at 192°C. and had the following composition.

CORRECTION.

0.0996 gm. substance: 0.2002 gm. CO_2 and 0.0446 gm. H_2O .

0.0927 " " : 20.60 cc. nitrogen gas at $t^\circ = 23^\circ\text{C}$. and $p = 762.4$ mm.

$\text{C}_{10}\text{H}_{10}\text{N}_4\text{O}_2$. Calculated. C 55.02, H 4.62, N 25.69.

Found. " 55.03, " 5.03, " 25.68.

4,5-Di(phenylhydrazino)uracil.—This was prepared from isobarbituric acid. It consisted of microscopic prismatic needles somewhat longer than the previous compound and had a very light orange color. The melting point was 160°C . and the substance analyzed as follows:

0.1004 gm. substance: 0.2196 gm. CO₂ and 0.0436 gm. H₂O.
 0.0958 " " : 21.70 cc. nitrogen gas at $t^{\circ} = 23^{\circ}\text{C.}$ and $p = 755.9$ mm.

C₁₃H₂₂N₆O₂. Calculated. C 60.97, H 6.26, N 23.73.
 Found. " 61.05, " 6.00, " 23.52.

Diphenylhydrazinouridine.—The substance had the appearance of a glucosazone. The crude material is easily recrystallized from 75 per cent ethyl alcohol or from methyl alcohol. The pure material, as already noted by Levene and La Forge, is very insoluble. It required 1 liter of boiling methyl alcohol to recrystallize 1.0 gm. of the substance. It can also be recrystallized from isobutyl alcohol. The substance melts at 212°C. and has the following composition.

0.1018 gm. substance: 0.2056 gm. CO₂ and 0.0468 gm. H₂O.
 0.0948 " " : 15.2 cc. nitrogen gas at $t^{\circ} = 24^{\circ}\text{C.}$ and $p = 765$ mm.
 C₂₁H₂₃N₆O₂. Calculated. C 55.23, H 5.30, N 18.42.
 Found. " 55.34, " 5.14, " 18.55.

A substance of the same composition and of the same melting point is formed if in place of uridine, bromouridine is employed. It is, however, necessary to proceed exactly as in the case of uridine. When bromouridine is treated directly with sodium acetate and phenylhydrazine, the derivative is not formed. Levene and La Forge described the method of preparation of 5-hydroxyuridine. If the crude material is treated with phenylhydrazine, the identical diphenylhydrazino derivative is formed.

The following substances did not form crystalline derivatives of phenylhydrazine: 5-methyluracil, 4-methyluracil, and cytidine.

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STUDIES ON RACEMIZATION.

ACTION OF ALKALI ON DEXTRO-ALANYL-DEXTRO-ALANINE ANHYDRIDE.

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(Received for publication, February 27, 1925.)

In 1906 Levene and Wallace and Levene and Beatty isolated *d*-prolyl-glycine anhydride from the products of tryptic digestion of gelatin. The question which immediately presented itself was whether or not ketopiperazides could be primary constituents of the protein molecule. Levene and Kober and Levene and Meyer approached the problem by the biological method. They followed the nitrogen partition in the urine of dogs to the diet of which one of the following four groups of substances was added: (1) amino acids, (2) dipeptides, (3) proteins, and (4) ketopiperazides. When one of the first three groups of substances was added, practically all the excess nitrogen was removed in the form of urea. When glycyl-glycine anhydride was added, all the excess nitrogen appeared in the form of undetermined nitrogen and not of urea.

It was then concluded that the catabolism of proteins in the organism of a dog was different from that of ketopiperazides. However, it was realized that within the protein molecule the ketopiperazide may be less stable than in a free state. Nevertheless, the result of the experiment cast doubt on the presence of ketopiperazines in the protein molecule. At the time, the problem aroused little interest among protein chemists. Also one of the writers (L.), at the time, failed to discover a satisfactory approach towards the solution of the problem and the work was then discontinued.

Very recently the problem came to the fore. Recent work on polysaccharides, work on x-ray analysis, and new observations on protein hydrolysis caused protein workers to think of the protein molecule as composed of comparatively simple anhydrides

of peptides held together by forces other than those of the primary valences. Hess, Herzog, Abderhalden, Ssadikow and Zelinsky, and, lately, Max Bergmann took an active interest in the problem. Brigl challenged the experimental evidence of Ssadikow and Zelinsky; namely, he demonstrated that under the conditions of the experiments of the latter, ketopiperazides may form from amino acids or peptides. The formation of ketopiperazides from peptides through the action of very dilute acids was demonstrated in a more quantitative way by Levene, Simms, and Pfaltz and Levene and Simms. These investigators showed that the same reaction takes place through the action of erepsin. These observations naturally counsel caution in forming conclusions regarding the presence of ketopiperazides in the protein molecule.

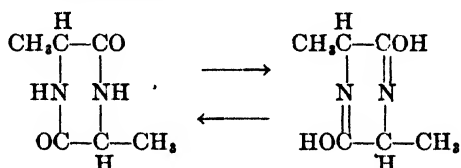
The fact that ketopiperazides are not hydrolyzed even by erepsin, which digests peptones and most simple peptides, also adds to the sum of evidence for the negative side of the question.

Recently an observation was made in this laboratory, which, when extended to larger material, may prove of assistance for the solution of the problem.

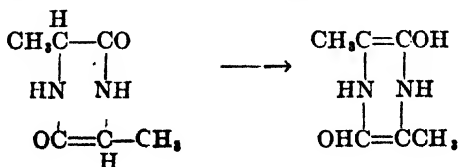
The observation is the following. When dextro-alanyl-dextro-alanine-anhydride is dissolved in normal alkali at $+3^{\circ}\text{C}$. (the concentration of the anhydride being 1 per cent), then the specific rotation of the substance is $+17.5^{\circ}$. The specific rotation in water is -28° . The rotation of the alkaline solution even at 0°C . drops very rapidly, reaching an equilibrium of -16° in less than 2 hours. When this equilibrium is reached the amino nitrogen of the solution is 50 per cent of the total. Thus, apparently, most of the anhydride is converted into the peptide.

If this solution which contains not more than a small portion of the anhydride and principally the peptide is allowed to stand 24 hours at $20-25^{\circ}\text{C}$. or even 72 hours at 12°C ., neither the optical activity nor the composition of the solute is changed. If the solution is then concentrated and made to contain 10 per cent of hydrogen chloride and hydrolyzed in a sealed tube for 12 or 18 hours at 100°C ., the solute acquires a specific rotation of $+14^{\circ}$, which is practically that of dextro-alanine. The specific rotation of dextro-alanine in a 10 per cent solution of hydrochloric acid is $+15^{\circ}\text{C}$.

From this experiment it follows that a dipeptide in a solution of normal alkali undergoes comparatively little racemization. Furthermore, if the original change of rotation from -27.5° in water to $+17.5^\circ$ in 1 N alkali is due to a tautomeric change, the character of this change is such that it does not lead to a racemization and may consist in a change of the ketopiperazide into a dioxy-dihydropyrazine.



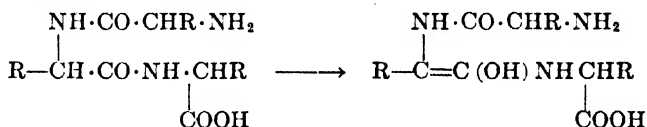
If the same ketopiperazide is taken up in a solution containing only 1 equivalent of alkali and the concentration of the anhydride remains 1 per cent, then the original change in optical rotation is minimal; namely, at 0°C ., the specific rotation of the solute is -25.5° instead of -27.5° in pure water. At $+3^\circ\text{C}$., the rotation remains constant for a considerable time (8 hours), but at the temperature of $20-25^\circ\text{C}$., it drops slowly and reaches an equilibrium after 24 hours. When the equilibrium is reached the solution contains 50 per cent of the total nitrogen in the form of amino nitrogen. The specific rotation of the solute is then -4° . Neutralization does not alter the specific rotation of the solution. When the solution is made to contain 10 per cent hydrogen chloride and is hydrolyzed for from 12 to 18 hours in a sealed tube at 100°C ., then the specific rotation of the solute is $+5^\circ$. Also in this case, all the nitrogen is present in the form of amino nitrogen. Thus it follows that only when the hydrolysis of the anhydride into the peptide proceeds slowly, so that a greater portion of the anhydride remains in solution for an appreciable time, does the substance undergo racemization. It is possible that under these conditions a different tautomerism takes place which leads to racemization; namely, the following.



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It is noteworthy that in experiments with stronger alkalies, the rate of change of rotation is practically equal to the rate of hydrolysis, whereas in those with weak alkalies, the rate of optical change is greater than the rate of hydrolysis. Therefore, under the influence of weak alkalies, racemization apparently precedes hydrolysis.

The bearing these observations may have on the theories of protein structure is the following. Kossel and Weiss have observed that when proteins are treated with dilute alkali (0.5 N), a gradual drop in their optical activity is observed. The rotation reaches a minimum and then remains constant. They recognized in the phenomenon a racemization for the reason that on hydrolysis the partially inactivated proteins gave rise to a certain proportion of *dl*-amino acids. Dakin and Dakin and Dudley extended these observations and noted that the degree of inactivation is characteristic for individual proteins and Dakin explained the mechanism of inactivation by a tautomeric change in the peptide.



From the present observations it is seen that for the mechanism of racemization of proteins an alternative explanation may be given; namely, that racemization of proteins is due to the presence in them of ketopiperazides. A decision in favor of one or the other possibility has to be postponed until the study is extended from dipeptides to polypeptides. If it should be found that polypeptides on treatment with alkali react in the same way as dipeptides, then the fact of racemization of proteins will stand as evidence in favor of the assumption of the presence of ketopiperazides in the protein molecule. On the other hand, if tripeptides and polypeptides react to the treatment with alkali in the same manner as ketopiperazides, then the present observations will have no bearing on the theory of the structure of the protein molecule but will serve to substantiate the theory of Dakin that in polypeptides only those amino acids are racemized which do not occupy the two terminal positions of the chain of amino acids.

EXPERIMENTAL PART.

The experiments were performed principally with dextro-alanyl-dextro-alanine anhydride. This was prepared from the ethyl ester. In 1 per cent aqueous solution the specific rotation was -27.5° . Herein is reported only one set of experiments of many, all of which proceeded with remarkable uniformity.

Behavior of Dextro-Alanyl-Dextro-Alanine in Solution of Strong Alkali.—The substance (0.2000 gm.) was dissolved in 20 cc. of 1 N sodium hydroxide solution at $+3^{\circ}\text{C}$. In all determinations of the specific rotation the temperature was kept constant by circulating constant temperature water through the jacket of the polariscope tube. The course of change of rotation is given in Table I.

In another experiment the solution was allowed to stand 3 days at 12°C . and the equilibrium rotation remained constant throughout that time.

After equilibrium was obtained the solution was neutralized with an equal volume of normal acid. The rotation in half the previous concentration was -0.14° . This solution was then concentrated and made up to a volume of 10 cc. and to a 10 per cent concentration of hydrogen chloride. It was heated in a sealed tube at 100°C . for 12, and, in some experiments, for 18 hours. The rotation calculated for the original concentration was $+28^{\circ}$. A 1 per cent solution of dextro-alanine in a 2 dm. tube rotates $+31^{\circ}$.

Rate of Hydrolysis of the Alanyl Alanine Anhydride into the Peptide.

It is seen from Tables I and II that the rotation equilibrium and hydrolysis equilibrium are established approximately at the same time. The rate of hydrolysis lags slightly behind that of mutarotation, and the equilibrium of the latter is established only a little ahead of the completion of the hydrolysis of the anhydride into the peptide.

The solutions used for measuring the mutarotations were neutralized, concentrated, and made up to a volume of 10 cc., containing 10 per cent of hydrogen chloride gas. Such solutions were hydrolyzed for 12 to 18 hours. At the end of the experiment the total and amino nitrogen were determined. In a

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TABLE I.

Time.	α	$a - x$	$K = \frac{1}{t} \log \frac{a}{a-x} \times 10^4$
<i>min.</i>			
0	+0.35		
15	+0.02	0.34	190
29	-0.10	0.22	166
48	-0.23	0.09	181
69	-0.28	0.05	184
90	-0.32		180 = average.
(equilibrium).			

$l = 2 \text{ dm.}; a = 0.67; t^\circ = +3^\circ\text{C}.$

TABLE II.

Time.	Amino N* in 20 cc. of solution.	$a - x$	$K = \frac{1}{t} \log \frac{a}{a-x} \times 10^4$
<i>min.</i>			
15	0.0069	0.0130	123
31	0.0130	0.0069	148
46	0.0147	0.0053	130
91	0.0190	0.0009	148
136	0.0199		139 = average.

$l = 2 \text{ dm.}; a = 0.0199; t^\circ = 0^\circ\text{C}.$

* This was measured by estimating at given intervals the amino nitrogen content of the solution. The micro method of Van Slyke was used.

TABLE III.

Time.	α	$a - x$	$K = \frac{1}{t} \log \frac{a}{a-x} \times 10^4$
<i>min.</i>			
0	-0.49		
32	-0.38	0.31	41.2
63	-0.30	0.23	41.5
106	-0.23	0.16	39.5
158	-0.16	0.09	42.4
Next day (equilibrium).	-0.07		41.5 = average.

$l = 2 \text{ dm.}; a = 0.42; t^\circ = +21^\circ\text{C}.$

typical experiment, 1 cc. of the solution contained 0.0035 gm. of total nitrogen and 0.0036 gm. of amino nitrogen (after 15 minutes of shaking in a Van Slyke apparatus). The results of the experiments were all uniform.

Thus it is seen that on treatment with a normal solution of sodium hydroxide dextro-alanyl-dextro-alanine anhydride is completely hydrolyzed into the peptide without perceptible racemization having taken place.

Behavior of Dextro-Alanyl-Dextro-Alanine Anhydride in Dilute Alkali.—The substance (0.2000 or 0.3000 gm.) was dissolved in 20 cc. of water containing exactly 1 equivalent of alkali and the mutarotation was observed at 20°C. The temperature was

TABLE IV.

Time.	Amino N in 20 cc. of solution.	$\alpha - x$	$K = \frac{1}{t} \log \frac{\alpha}{\alpha - x} \times 10^4$
<i>min.</i>			
60	0.0028	0.01657	11.3
120	0.0056	0.01379	10.6
180	0.0073	0.0121	11.9
240	0.0090	0.0104	11.3
300	0.0117	0.0077	13.3
360	0.01264	0.0067	12.7
Next day.	0.01937		11.8 = average.

$l = 2$ dm.; $\alpha = 0.01937$; $t^\circ = +21^\circ\text{C}$.

maintained constant throughout the experiment. The course of the reaction is given in Table III. At 0°C. the mutarotation remained constant for over 3 hours and hence the measurement at that temperature was not continued.

At the end of the experiment the solution was accurately neutralized. The optical rotation remained unchanged. The solution was then concentrated and made up to a volume of 10 cc. of a solution containing 10 per cent of hydrogen chloride gas. The solution was then hydrolyzed in a sealed tube for 12 hours at 100°C. At the end of the experiment the rotation was $+0.08^\circ$. In the corresponding experiment with normal alkali the rotation was $+0.28^\circ$, thus showing a marked degree of racemization through the action of alkali at a low concentration.

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Rate of Hydrolysis of Dextro-Alanyl-Dextro-Alanine Anhydride into Its Peptide through the Action of Alkali of Low Concentration.

From the comparison of Tables III and IV it is seen that the rate of change of rotation is considerably higher than that of hydrolysis of the anhydride into the peptide, thus indicating that the process of racemization precedes that of hydrolysis.

When the equilibrium solution was hydrolyzed in a 10 per cent solution of hydrochloric acid for 12 hours at 100°C. all the nitrogen was accounted for by the amino nitrogen.

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ON SPHINGOSINE.

V. THE SYNTHESIS OF 1-AMINO-2-HYDROXY-*n*-HEPTADECANE.

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(Received for publication, February 28, 1925.)

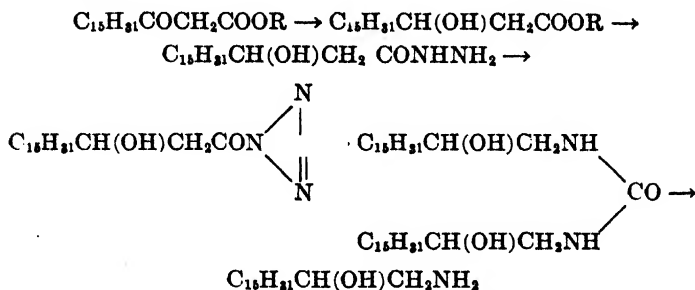
Through the work of recent years, many details of the structure of sphingosine have been explained; however, some of them still remain to be disclosed. It has been established that the base is a monoamino dihydroxy-*n*-heptadecylene. It was shown that the double bond is situated between carbon atoms 13 and 14, and that the three active groups substitute carbon atoms 15, 16, and 17. The allocation of the amino group and the configuration of the two respective asymmetric carbon atoms remain to be established. The problem was simplified by the fact that it was possible to reduce sphingosine to the base sphingine which is an amino hydroxy-*n*-heptadecane. There is reason to believe that the two active groups substitute carbon atoms 1 and 2. The evidence will be presented in a separate publication. The allocation of the amino group still remains unknown. An attempt has now been made to solve the problem by synthesis.

Levene and Scheidegger¹ have recently shown that it is possible to prepare the lower hydroxyamines by the method employed by Curtius for the preparation of simple amines. The method was now successfully applied to the preparation of *dl*-1-amino-2-hydroxy-*n*-heptadecane. After this base is resolved into its optical components, it will be possible to compare its properties with those of sphingine. On the basis of this comparison it should be possible to determine the allocation of the amino group in sphingosine.

Incidentally this work led to the first synthetic preparation of the 3-hydroxystearic acid (β -hydroxystearic) and thus the constants given in the literature for that substance were verified.

¹ Levene, P. A., and Scheidegger, J., *J. Biol. Chem.*, 1924, ix, 179.

The steps involved in the synthesis of the new hydroxyamine are as follows:



EXPERIMENTAL.

The palmitic acid used in the following experiments was purified by fractionation of the methyl ester of a commercial product. The free acid was obtained in the usual way and recrystallized several times from acetone. It melted at 63°C. and analyzed as follows:

0.0998 gm. substance: 0.2736 gm. CO₂ and 0.1120 gm. H₂O.
 C₁₆H₃₂O₂. Calculated. C 74.91, H 12.58.
 Found. " 74.76, " 12.55.

Palmityl chloride was prepared from palmitic acid and thionyl chloride. The acid chloride was purified by distillation under diminished pressure. It boiled at 155–160°C. (*p* = 0.2 mm.).

0.1248 gm. substance: 0.01615 gm. Cl.
 C₁₆H₃₁OCl. Calculated. Cl 12.93.
 Found. " 12.94.

Ethyl Palmityl Acetoacetate, CH₃CO·CH (COC₁₅H₃₁) CO·OC₂H₅.
 —To 2.3 gm. of finely divided sodium suspended in 300 cc. of dry ether, 26 gm. of ethyl acetoacetate were slowly added. After standing overnight all the sodium was in solution. 27.5 gm. of palmityl chloride were then slowly dropped in, the flask being frequently shaken. The sodium derivative of ethyl acetoacetate soon went into solution. Sodium chloride settled out when most of the palmityl chloride has been added. After the addition of all the acid chloride, the reaction mixture was allowed to stand overnight or it was heated to boiling under reflux, with exclusion of air, on

the water bath for 1 hour. The ethereal solution was washed with water to remove the sodium chloride, and then dried over anhydrous sodium sulfate. The ether and most of the excess ethyl acetoacetate were removed by distillation under diminished pressure. The palmityl acetoacetic ester was recrystallized several times from 95 per cent alcohol. It melted at 36–36.5°C.² The yield was 23 gm. or 62 per cent of the theory.

0.1011 gm. substance: 0.2658 gm. CO₂ and 0.1012 gm. H₂O.

C₂₂H₄₀O₄. Calculated. C 71.68, H 10.94.

Found. " 71.69, " 11.20.

Ethyl Palmityl Acetate,² C₁₅H₃₁COCH₂COOC₂H₅.—20 gm. of ethyl palmityl acetoacetate were dissolved in 180 cc. of water containing 1 equivalent of sodium hydroxide. The solution was heated on a boiling water bath for 45 minutes. The milky solution was rapidly cooled and the precipitate which had formed was filtered off. The precipitate was dissolved in ether and the solution washed first with a 10 per cent and then with a 20 per cent solution of sodium carbonate. After drying the ethereal solution over sodium sulfate, the ether was removed and the ethyl palmityl acetate was recrystallized several times from 95 per cent alcohol. The yield was 11 gm. or 62 per cent of the theory. The substance melted at 37–38°C.

0.1010 gm. substance: 0.2734 gm. CO₂ and 0.1060 gm. H₂O.

C₂₀H₃₈O₂. Calculated. C 73.55, H 11.74.

Found. " 73.81, " 11.74.

3-Hydroxystearic Acid, C₁₅H₃₁CH(OH)CH₂COOH.—10 gm. of ethyl palmityl acetate were dissolved in 800 cc. of 75 per cent alcohol and 20 gm. of 2.5 per cent sodium amalgam were added. The mixture was shaken in a shaking machine at room temperature. When all the sodium had gone into solution, the solution was neutralized with glacial acetic acid. An additional 20 gm. of amalgam were added and the above described process was repeated until 240 gm. of amalgam had been added. The slightly alkaline solution was concentrated to dryness, the residue treated with dilute hydrochloric acid and extracted with ether. After removing the ether, the residue was saponified in 90 per cent alcohol

² Helferich, B., and Köster, H., *Ber. chem. Ges.*, 1923, lvi, 2090.

with excess sodium hydroxide in the usual way. The alcoholic soap solution was poured into acetone, the soap filtered off and washed several times with acetone. The soap was decomposed with dilute hydrochloric acid.

After crystallizing the free acid from acetone, it was repeatedly washed with petroleum ether (boiling point 40–60°C.) and finally recrystallized from acetone. The acid melted at 90°C. Ponzio³ who prepared this acid from 2,3-oleic acid found 89°C.

Ethyl 3-Hydroxystearate, $C_{15}H_{31}CH(OH)CH_2COOC_2H_5$.—10 gm. of 3-hydroxystearic acid were dissolved in 100 cc. of absolute ethyl alcohol containing 3 per cent sulfuric acid. The solution was refluxed for 7 hours on the water bath, then placed in the refrigerator. The ester crystallizes slowly. It was filtered off and the mother liquor concentrated under diminished pressure to about 60 cc. and placed in the refrigerator at 3°C. A second crop of crystals was obtained which was combined with the first. The ester was crystallized from a small amount of absolute ethyl alcohol and then from acetone. It melted at 46°C.

0.0996 gm. substance: 0.2668 gm. CO_2 and 0.1088 gm. H_2O .

$C_{20}H_{40}O_3$. Calculated. C 73.10, H 12.27.

Found. " 73.05, " 12.22.

3-Hydroxystearylhydrazide, $C_{15}H_{31}CH(OH)CH_2CONHNH_2$.—6.6 gm. of ethyl 3-hydroxystearate were added to 2 gm. of hydrazine hydrate in a round bottom flask fitted with a ground glass air condenser. The mixture was heated over a flame until it melted and maintained in that condition for 8 hours with frequent shaking. It was then placed on the steam bath overnight. The white solid mass was transferred to a Büchner funnel with the aid of a small amount of absolute ethyl alcohol. Two recrystallizations from alcohol gave a pure product. On heating, the hydrazide gradually softens and melts at 123–124°C. with some decomposition. The yield was 6.5 gm. or 93 per cent of the theory.

0.1000 gm. substance: 7.9 cc. nitrogen gas at 23°C. and 758.0 mm.

0.1014 " " : 0.2552 gm. CO_2 and 0.1114 gm. H_2O .

$C_{15}H_{31}O_2N_2$. Calculated. C 68.74, H 12.18, N 8.92.

Found. " 68.63, " 12.23, " 9.08.

³ Ponzio, G., *Gazz. chim. ital.*, 1905, xxxv, pt. 2, 132.

3-Hydroxystearylhydrazide Hydrochloride.—This is prepared by suspending the hydrazide in dry ether and passing dry HCl into the suspension. It may also be prepared by intimately mixing concentrated hydrochloric acid with the hydrazide. On heating the hydrochloride it gradually decomposes.

Sym.-2-Hydroxyheptadecylurea, $(C_{15}H_{31}CH(OH)CH_2NH)_2 CO$.—2 gm. of hydroxystearylhydrazide hydrochloride were made into a paste with water and spread on the walls of a beaker and the mixture was cooled in ice water. A slow stream of nitrous acid (prepared from arsenious oxide and nitric acid, sp. gr. 1.32) was passed into the mixture. The mass soon became fat-like and floated on the water. It was filtered off and washed, first with water, then thoroughly with 2 per cent sodium carbonate solution, and finally with water. After drying it was extracted with ether. Most of it went into solution. The ether-insoluble portion consists principally of unchanged hydrazide. The ether-soluble fraction was concentrated to dryness under diminished pressure, and the residue twice recrystallized from absolute ethyl alcohol. Melting point 94°C.

0.0990 gm. substance: 4.2 cc. nitrogen gas at 24°C. and 754.3 mm.

0.0990 " " : 0.2668 gm. CO₂ and 0.1906 gm. H₂O.

C₃₅H₇₂O₃N₂. Calculated. C 73.8, H 12.7, N 4.9.

Found. " 73.5, " 12.38, " 4.83.

2-Hydroxyheptadecylamine, $C_{15}H_{31}CH(OH)CH_2NH_2$.—The above described urea derivative was heated with 20 per cent sulfuric acid in a sealed tube for 10 hours at 125°C. After cooling, the reaction mixture was filtered off, dissolved in a small amount of alcohol, and precipitated with cold ether.

20 mg. substance: 1.51 cc. nitrogen gas (Van Slyke) at 23°C. and 768.0 mm.

0.0992 gm. substance: 0.2322 gm. CO₂ and 0.1058 gm. H₂O.

C₃₅H₇₂O₃N₂. Calculated. C 63.69, H 11.96, N 4.36.

Found. " 63.77, " 11.93, " 4.28.

THE ISOLATION OF CRYSTALLINE ADENINE NUCLEOTIDE FROM BLOOD.

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(Received for publication, February 25, 1925.)

In 1914, Bass (1) found that the purine bases in normal blood were present in combined form. He was able to identify one of these combined purines as adenine, and suggested its existence in blood as adenylic acid (adenine nucleotide?).

More recently, Jackson (2) has isolated from blood a uranyl compound, which he has shown to contain adenine and easily hydrolyzable phosphoric acid, to give pentose color reactions, and to behave in general as one would expect the uranyl salt of adenine nucleotide to behave. In a later paper (3) he has obtained, as a crude amorphous substance, the compound or compounds which he had presumably studied as the uranyl salt in his first paper. With the indirect evidence of the first paper, a further proof of the presence of adenine, a total nitrogen and phosphorus analysis on the crude product, along with a determination of partial phosphorus and approximate adenine yield, Jackson concludes that he has obtained "either the so called adenine uracil dinucleotide or what is far more likely, a mixture of nucleotides."

Jackson's presumption of having uracil nucleotide can be dismissed with a word. Aside from his total and partial phosphorus analyses, figures which might be given by any one of an indefinite number of combinations of phosphorus-containing compounds, he has no evidence at all of the presence of a pyrimidine nucleotide, and certainly none for the presence of uracil nucleotide proper.

Jackson's indirect indication, however, of the presence of adenine nucleotide, is much more convincing. And yet, even if one assumes that the reactions which Jackson obtained could not,

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with any degree of probability, have been given by a mixture of compounds, there still remains the possibility of his having found a substance similar to but not identical with adenine nucleotide. It is evident, then, that conclusive proof of the presence of adenine nucleotide in blood can be shown only by the isolation of the pure crystalline nucleotide itself, as Jackson himself states.

It was with this aim that the present research was undertaken. Since the amount of human blood required to obtain a pure analyzable product would be entirely impossible to procure, pig's blood was used as an alternative representative of mammalian blood. Large quantities of pig's blood were deproteinized and treated for nucleotides along the general methods used for obtaining nucleotides or nucleic acid from tissues. In principle, the process is quite simple. It consists of precipitating the deproteinized blood with lead acetate in faintly acid solution, removing the lead from the precipitate by means of hydrogen sulfide, evaporating the resulting solution to a syrup under diminished pressure, and hardening the syrup by means of absolute alcohol. The amorphous substance thus obtained will contain the adenine nucleotide present in the blood, which can be purified by recrystallization of its brucine salt from 35 per cent alcohol. From the brucine salt, crystalline adenine nucleotide can be prepared in the usual way through the lead salt.

By this method, there was obtained a crystalline substance which, in its chemical analysis and its physical and chemical properties, is identical with adenine nucleotide obtained by Jones and Kennedy from yeast nucleic acid (4). It exhibits supersaturation to a marked degree, but finally crystallizes from water in characteristic needle clusters. It contains 1 molecule of water of crystallization which it loses sharply at 110° and takes up again quantitatively on exposure to air at ordinary temperatures. It responds in minutest amounts to the pentose color reactions, contains the amount of nitrogen and phosphorus required for crystalline adenine nucleotide, and on hydrolysis with dilute sulfuric acid produces adenine, but not a trace of guanine. The adenine is unmistakably identified as the picrate, which crystallizes in long pale yellow needles, resembling matted hair. The picrate melts with decomposition in a capillary tube at about 280°C . There is accordingly, no doubt that the substance obtained from the pig's blood is adenine nucleotide.

EXPERIMENTAL.

Deproteinization of the Blood.—The pig's blood was worked up in batches of 10 liters each. The first problem, that of deproteinization, was not a simple one. Tungstic acid as a deproteinizer, was avoided, on the strength of Jackson's warning. Picric acid, which he used, was also found unsatisfactory, since it is partially precipitated with the lead nucleotide and produces difficulties in the isolation of the nucleotide. Sulfosalicylic acid, recommended by Thannhauser and Czoniczer (5), was found to hydrolyze a large fraction of the nucleotide. The method of deproteinization finally adopted, with modifications, was that used by Davis, Newton, and Benedict (6) in the isolation of uric acid pentose from blood.

1.5 liters of fresh defibrinated pig's blood were added to 7.5 liters of boiling water containing 5 cc. of glacial acetic acid and 15 gm. of sodium acetate crystals. The solution was allowed to continue boiling for a minute or so, and was then filtered immediately through large plaited filters. The presence of the sodium acetate in the solution produces a very rapid filtration. The yellowish transparent filtrate was concentrated on the water bath in large dishes with the aid of a fan. 10 liters of blood, deproteinized with 50 liters of acetic acid solution, were allowed to concentrate to a volume of 3 liters and were then refiltered.

This solution still contains a small amount of protein. To remove these last traces, Davis, Newton, and Benedict recommend the use of colloidal ferric hydroxide. When this was tried, it was found that not only was the protein precipitated but along with it every trace of nucleotide. It was this fortunate circumstance which permitted Davis, Newton, and Benedict to isolate uric acid pentose without the interference of nucleotides. In the present problem, it was found best not to attempt to remove these last traces of protein.

Preparation of the Crude Nucleotide.—To the concentrated solution was added a large excess of saturated lead acetate followed by ammonium hydroxide to alkaline reaction. The precipitate obtained was filtered off, and washed with cold water. It was then suspended in hot water containing a little sodium acetate, and treated with hydrogen sulfide until a small portion of the filtered fluid gave no more blackening with hydrogen sulfide.

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The presence of the sodium acetate prevented the formation of a colloidal solution of lead sulfide in the presence of the remaining protein. The lead sulfide was filtered off, and the filtrate, from which the excess of hydrogen sulfide was removed by aeration, was reduced to faint acidity with ammonium hydroxide and reprecipitated hot with an excess of lead acetate. This lead precipitate was suspended in pure hot water, and treated as above with hydrogen sulfide to remove the lead. The solution obtained was protein-free. Apparently the protein had been adsorbed on the lead sulfide. This solution, after aeration, was evaporated to a syrup under diminished pressure at 50° and hardened by grinding with absolute alcohol. The amorphous powder obtained was dried in a desiccator. This product contains along with the adenine nucleotide all the substances present in deproteinized blood that are precipitable by lead acetate in faintly acid solution and are insoluble in absolute alcohol. An average yield of 2.1 gm. was obtained from 10 liters of blood.

Preparation of the Crystalline Nucleotide by Means of the Brucine Salt.

The crude nucleotide was dissolved in a minimum amount of hot water and was neutralized by an alcoholic solution of brucine. After spontaneous evaporation for several days, the solution finally gave a crystalline brucine salt, which was recrystallized from 35 per cent alcohol. This was suspended in hot water and treated with a slight excess of ammonia. On cooling, the brucine was precipitated and filtered off. The filtrate, which was extracted by chloroform to remove the last traces of brucine, was made faintly acid with acetic acid and treated in the hot with an excess of lead acetate. The lead salt was filtered off, washed thoroughly, and treated with hydrogen sulfide to remove the lead. The filtrate from the lead sulfide, after aeration, was allowed to evaporate spontaneously for several days, in which time there crystallized a white compound consisting of fine microscopic needle clusters. These crystals were filtered off, washed, and recrystallized from hot water.

Chemical Composition of the Nucleotide.—Analysis of the crystalline compound gave the following figures.

- I. 0.1094 gm. required 5.87 cc. of standard sulfuric acid (1 cc. = 0.003553 N).
 II. 0.0746 gm. required 4.08 cc. of standard sulfuric acid (1 cc. = 0.003553 N).
 III. 0.3454 gm. lost 0.0179 gm. at 110° and regained 0.0179 gm. on exposure to air.
 IV. 0.0746 gm. lost 0.0037 gm. at 110° and regained 0.0037 gm. on exposure to air.
 V. 0.2064 gm.: 0.1439 gm. $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$.

	Theoretical for $\text{C}_{10}\text{H}_{14}\text{N}_4\text{P}_2\text{O}_7 \cdot \text{H}_2\text{O}$.	I	II	III	IV	V
N...	19.18	19.06	19.43			
H ₂ O.	4.93			5.12	5.01	
P....	8.49					8.81

SUMMARY.

A crystalline substance has been isolated from pig's blood which is identical with crystalline adenine nucleotide obtained from yeast nucleic acid.

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CLINICAL CALORIMETRY.

XXXVIII. THE UTILIZATION OF CARBOHYDRATE IN A CASE OF RENAL GLYCOSURIA.

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INTRODUCTION.

To what extent an individual with renal glycosuria can oxidize glucose is a question upon which precise data are for the most part lacking. We present, herewith, data which were obtained in the study of such a case. They include observations made with the respiration calorimeter of the Russell Sage Institute of Pathology at Bellevue Hospital.

In a clinical and metabolic study of a patient with renal glycosuria, Finley and Rabinowitch (1) noted the effect of the ingestion of glucose on the respiratory quotient, and found a sharp rise to a maximum of 0.92, indicating marked increase in the oxidation of carbohydrate.

CLINICAL DATA AND RESULTS.

The patient, Sam D., Jewish, male, born in 1909, came under our observation first at about the age of 12 years. He has always appeared healthy and robust, well developed, and with good muscle tone. The sugar in his urine was discovered in the course of a routine examination when, shortly before we saw him, he was operated upon for acute appendicitis. His recovery was uneventful. Measles is the only other disease he has had except for occasional attacks of tonsillitis and colds. His mother is said to have had diabetes for several months, years ago, from which she recovered. Her urine is sugar-free.

Physical examination showed a healthy boy, well developed, with large cryptic tonsils from which plugs of pus could be expressed. Appendectomy scar present and neat. Otherwise negative.

The patient sought medical advice in regard to his supposed diabetes. Two periods of strict dieting are said to have had little if any effect on the glycosuria. Sugar has always been present in the urine whenever tested since discovery. He eats about what he pleases, avoiding large quantities of candy or sugar. He has continued to go to school or to work, is active, enjoying the pleasures of boys his age, and plays basket-ball. His habits are good.

During the 2 years of observation by us, his blood sugar has never been found to be over 1.8 gm. per liter and the urine has never been sugar-free nor the qualitative test less than 4+. There has been no thirst, polyuria, or polyphagia.

On June 19, 1922, his breakfast contained 25 gm. of protein, 30 gm. of fat, and 100 gm. of carbohydrate. It was eaten at 7.45 a.m. The blood sugar at 8.15 a.m. was 0.94; at 8.30 a.m., 1.40; at 9.00 a.m., 1.60 gm. per liter. The sugar excreted in the urine from 7.45 to 9.00 a.m. was 3.5 gm. On the same day his total diet was: protein 71, fat 100, carbohydrate 300, and the total urinary sugar for 24 hours was 36 gm.

On June 27, 1922, his breakfast consisted of 25 gm. of protein, 30 gm. of fat, and 130 gm. of carbohydrate, ingested at 7.45 a.m. The blood sugar at 8.30 a.m. was 0.95; at 9.00 a.m., 1.20; at 10.00 a.m., 1.03 gm. per liter. The sugar excreted in the urine during the 2 hours was 10.9 gm. The total diet for the day was: protein 70, fat 100, and carbohydrate 400 gm. The sugar excreted in the urine during these 24 hours was 39.1 gm.

On Oct. 10, 1923, the following observations were made.

Time.	Blood sugar.	Urine volume.	Glucose.
<i>p. m.</i>	<i>gm. per l.</i>	<i>cc.</i>	<i>per cent</i>
2.55	1.16 (Fasting.)	136	5.0
3.00 (Ingested 60 gm. glucose in water.)			
3.30	1.80	62	5.0
4.05	1.65	58	5.5
5.03	1.55	72	6.5

On Apr. 5, 1923, the patient was observed in the calorimeter. His age was then 14 years, and his height 157 cm. For two 1 hour periods his basal metabolism was determined. He then ingested, at the beginning of the 3rd hour, 150 gm. (3 gm. per kilo of body weight) of carbohydrate in the form of glucose in water flavored with lemon juice. The respiratory exchange was measured for three additional 1 hour periods.

The observations were controlled by means of alcohol checks, a table of which appears in another paper of this series (2). The principal data are shown in Table I.

TABLE I.
Effect of Ingested Glucose on the Respiratory Exchange and the Urinary Excretion in Renal Glycosuria.

Hour.	Respiratory exchange.							In urine per hr.			Remarks.
	Respiratory quotient.	Non-protein R. Q.	Indirect heat.	Total calories from			Work added.*	Nitrogen	Glucose.	Acetone.	
				Protein.	Fat.	Carbohydrate.					
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	
			cal. per hr.	per cent per cent per cent			cm.	gm.	gm.	gm.	
1	0.803	0.802	57.6				30				Slightly restless.
2	0.829	0.836	58.2				23				"
Average of 1 and 2.	0.816	0.819	57.9	20.8	47.5	31.7	27	0.455	1.13	Trace.	150 gm. glucose ingested during 16th to 19th min. Voided.
3	0.790	0.785	67.8	19.7	57.8	22.5	31	0.503	2.82	"	Restless.
4	0.859	0.872	63.6	18.8	33.9	47.3	60	0.450	4.15	0	Quiet.
5	0.926	0.966	52.3	22.8	8.4	68.8	13				
Following 13 hrs. 17 min.											
										1.6	

* The work adder is an instrument by which the activity of the patient is recorded. A very quiet patient raises the work adder less than 5 cm. and a very restless patient more than 25 cm.

It may be seen from Column 2 that the respiratory quotient averaged 0.816 for the 2 basal hours, fell to 0.790 in the hour in which food was taken, and then rose sharply reaching 0.926 in the last hourly period. If the figures be corrected for the influence of protein, the quotient of carbohydrate-fat oxidation is obtained. This is known as the non-protein respiratory quotient and appears in Column 3. In the last hour it rose to 0.966, showing oxidation of carbohydrate almost to the complete exclusion of fat.

The blood sugar before the observation was 1.0 gm. per liter. 30 minutes after the end of the last period it was 1.2 gm. per liter. The diet for the 24 hours contained 23 gm. of protein, 58 gm. of fat, and 200 gm. of carbohydrate. 150 gm. of the last consisted of the glucose in lemon juice above mentioned. The glucose in the urine was 36.5 gm. in 24 hours. That this was actually glucose was evidenced by the fact that it fermented readily with the evolution of a large volume of gas. Identification of the sugar as glucose was confirmed by further analyses which were very kindly performed by Dr. Stanley R. Benedict.

Comment.

The data presented here may be judged better in comparison with the effect of the ingestion of glucose by a normal individual, and by a patient who had exophthalmic goiter. The normal subject E. F. Du B. studied in Paper IV (3) of this series had a basal respiratory quotient of 0.84 to 0.80 and a basal non-protein quotient of from 0.85 to 0.80. After the ingestion of 200 gm. of glucose, or 2.7 gm. per kilo of body weight, his non-protein quotient rose on three occasions to unity, indicating oxidation of carbohydrate to the exclusion of fat. Max W. the patient with exophthalmic goiter, who was reported in Paper XIV (4) of this series, reacted in very much the same way to a smaller quantity of glucose; that is, 1.6 gm. per kilo of body weight. The peak was reached within 3 hours in this case, but not until 5 hours after ingestion in one of the observations with the normal individual.

This patient shows unusual and rather constant permeability of the kidneys to glucose with, at the same time, no symptoms of diabetes mellitus and a blood sugar always within normal limits

except in one instance (1.8 gm. per liter). He has continued in good health with these conditions present for at least 3 years. In March, 1924, he underwent tonsillectomy with ether anesthesia without undue reaction.

During the calorimetric observations a postprandial drop in the respiratory quotient is noted such as has been reported and discussed elsewhere (2). The cause is unknown and it is mentioned as of interest because of occurring in renal glycosuria.

SUMMARY AND CONCLUSION.

The data above presented show that a patient with renal glycosuria was able to utilize carbohydrate to the same extent as a normal individual. In other words, a moderate degree of permeability of the kidney to glucose can occur without any demonstrable defect in the oxidation of carbohydrate. As the power to oxidize glucose was not diminished, there appears to be no ground, so far as this patient is concerned, for believing that renal glycosuria is a preliminary stage of diabetes mellitus.

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THE TETANY OF PARATHYROID DEFICIENCY AND THE CALCIUM OF THE BLOOD AND CEREBRO-SPINAL FLUID.

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In 1923 Salvesen (1) confirmed the theory first put forward by MacCallum and Voegtlin (2) that calcium deficiency is the cause of the tetany following parathyroidectomy, and concluded from his experiments that the parathyroids control the calcium level of the blood, showing further that if sufficient calcium were given in the diet, whether normally in milk or by the addition of calcium salts, parathyroidectomized animals can be maintained under normal conditions for at least 2 years, though during this period the serum calcium does not appear to rise above 7 mg. per 100 cc. He found that whenever calcium-poor food was given such animals they developed tetany very quickly, with a corresponding fall in the serum calcium to 4 or 5 mg. He suggested that after removal of parathyroids there is a lowered threshold for the excretion of calcium in the intestine, and that this is apparently the cause of the calcium deficiency and the resultant symptoms.

In later papers Salvesen and Linder (3) found that in conditions such as nephritis with uremia diminished plasma protein was accompanied by a marked fall in the calcium content though no tetany resulted, while following parathyroidectomy the serum calcium decrease was not accompanied by diminution of serum protein. Hence they concluded that the lowering of serum calcium in parathyroid tetany is chiefly due to change in the "ionized calcium."

Edema fluid in certain of the nephritis cases, produced, it may be assumed, by natural dialysis, showed that from 55 to 70 per cent of the plasma calcium is diffusible, in agreement with the results of Rona and others.

We considered that information on the relative changes in the diffusible and non-diffusible plasma calcium following parathyroidectomy might be gained by a comparative study of the calcium present in serum and cerebrospinal fluid, since the latter is usually regarded as a (selected) filtered blood plasma.

The cerebrospinal fluid calcium is usually stated to be between 6 and 7 mg. per 100 cc., in *rough* agreement with the proportion of plasma calcium considered diffusible. A paper recently published by Critchley and O'Flynn (4), shows that the cerebrospinal fluid calcium is very constant, and only becomes distinctly lowered in tetany.

Since in tetany the serum calcium decreases from 10 to 11 mg. to 5 to 6 mg. or less, and since this fall was considered by Salvesen to affect the "ionizable calcium" chiefly, we expected to find that the calcium of the cerebrospinal fluid would almost completely disappear. We carried out an initial series of experiments on five dogs. Calcium was estimated in serum and cerebrospinal fluid by Tisdall's modification of Kramer and Tisdall's method (5). It may be pointed out that, using 2 cc. of fluid for analysis, an error of 1 drop of 0.005 N permanganate gives an error of 0.2 mg. per 100 cc. of fluid, while if 1 cc. of fluid is used, this error is doubled. While we have endeavoured to obtain a correct end-point to within half a drop of permanganate, this must frequently have been exceeded. The error arising from permanganate requirement for the actual amount of liquid used in the titration is entirely offset by the opposite error of loss of calcium oxalate during washing, so that the actual titration figures have been used. Using Clark's calculation,¹ the calcium oxalate dissolved and lost while washing with 12 cc. of water—we have used in all 12 cc. of dilute ammonia for the three washings employed—would be, if saturation were complete, 0.02 mg. The excess permanganate required for the amount of liquid used in the titration has been ascertained by blank experiments to be about 0.008 mg. Hence the figures for calcium that we have actually obtained and used are, without correction, very slightly too low by a constant amount.

Before dealing with the results following parathyroidectomy the figures for serum and cerebrospinal fluid calcium of normal dogs determined during the whole period of the research, may be summarized, and are given in Table I. We shall refer later to the possible slight abnormality of some of these dogs, leading perhaps to a certain degree of dehydration (which may account for such a result as that with Dog 7).

In Table I serial numbers are given to the dogs subsequently

¹ Clark (6), p. 499.

TABLE I.

Relation between the Cerebrospinal Fluid and Serum Calcium of Normal Dogs.

Dog No.	Date.	Serum.			Cerebrospinal fluid.			Cerebrospinal Ca Serum Ca
		Volume.	Ca per 100 cc.		Volume.	Ca per 100 cc.		
				Mean.			Mean.	
	1924	cc.	mg.	mg.	cc.	mg.	mg.	
A	Jan. 11	2	12.7		2	6.2		
		1	12.5	12.6	1	6.0	6.1	0.48
B	" 11	2	12.1		2	5.5		
		—	—	12.1	2	5.7	5.6	0.46
C	" 15	2	11.7		1	4.8		
		1	11.2	11.5	1	4.8	4.8	0.42
1	" 19	1	13.5		1	6.9		
		1	12.1	12.8	1	6.6	6.8	0.53
2	" 22	2	11.4		1	7.7		
		1	11.5	11.4	—	—	7.7	0.68
3	" 26	2	13.0		2	8.0		
		2	11.9	12.5	2	7.5	7.8	0.62
4	" 29	2	12.4		2	6.8		
		2	11.7	12.1	2	6.4	6.6	0.55
5	Feb. 5	2	10.8		1	5.6		
		1	10.3	10.6	—	—	5.6	0.53
6	Nov. 17	1	10.9	10.9	1	4.9	4.9	0.45
7	" 24	2	8.6		1	6.3		
		2	8.6	8.6	—	—	6.3	0.73
N	Dec. 15	2	12.5		2	5.4		
		2	12.4	12.5	2	5.4	5.4	0.43
D	" 15	2	10.2		2	5.1		
		2	10.5	10.4	2	6.0	5.6	0.54
9	" 17	2	10.4		2	5.3		
		2	10.8	10.6	2	5.6	5.5	0.52
	" 22	2	9.9		2	5.5		
		2	10.1	10.0	2	5.5	5.5	0.55
E	" 20	2	8.9		2	4.2		
		2	8.9	8.9	2	4.5	4.4	0.49
F	" 30	2	10.7		2	5.8		
	1925	2	10.7	10.7	2	5.8	5.8	0.54
G	Jan. 2	2	10.0		2	5.1		
		2	10.2	10.1	2	5.2	5.2	0.51
10	" 9	2	11.2		2	5.5		
		2	11.4	11.3	2	5.6	5.6	0.50
	" 12	2	10.0		2	4.8		
		2	10.0	10.0	2	4.8	4.8	0.48
L	" 21	2	11.0		2	6.2		
		2	11.2	11.1	1	6.0	6.1	0.55
11	" 28	2	12.2		2	6.1		
		2	12.4	12.3	—	—	6.1	0.50
12	Feb. 4	2	11.3		2	6.3		
		2	11.1	11.2	1	6.2	6.3	0.56
Averages (22 determinations on 20 dogs).....				11.1			5.8	0.53

parathyroidectomized. The results are in general agreement with previously published data, and show, for most animals, a fairly constant ratio between the calcium content of the two fluids. They also indicate that fair reliance can be placed on results for 1 cc. samples when more material is not available (as has been the case sometimes with the parathyroidectomized dogs). It is to be noted that the cerebrospinal fluid calcium averages only 53 per cent of the serum calcium, a figure distinctly less than the amount of "dialyzable calcium" of the serum ascertained by compensation-dialysis methods.

Operative Procedure.

Dogs were used entirely in these experiments. The normality of such dogs is variable, since they were obtained during the severe winters of this climate, and some of them had been running loose on the streets. They may in consequence have been in a condition of malnutrition and even dehydration. In most cases they were kept some days under observation before operation. Dogs with obvious defects were discarded, and only healthy animals employed.

Parathyroid removal was carried out by the operation of complete thyroparathyroidectomy, except with Dog 3, in which right thyroparathyroidectomy and left parathyroidectomy were performed. Blood samples were taken from the external jugular or the femoral vein or their branches. Cerebrospinal fluid was collected from a needle inserted into the cisterna magna. Ether anesthesia was used in taking these fluids, because of difficulties with local anesthesia in taking large amounts.

After thyroparathyroidectomy the dogs usually developed tetany in from 1 to 5 days. Blood and cerebrospinal fluid samples were then taken, and in some instances the dogs were kept free from tetany by subcutaneous administration of calcium chloride or lactate and a milk diet. When desired these dogs could be caused to develop marked tetany by completely withholding calcium. Our results in such cases are in agreement with Salvesen's observations, though more frequent calcium injection has been necessary. Since adult dogs were always used we have assumed that there should be no marked effects from thyroid deficiency during the relatively short experimental period.

TABLE II.
First Series of Parathyroidectomized Dogs.

Dog No.	Time since operation.	Serum.			Cerebrospinal fluid.			Remarks.
		Vol- ume.	Ca per 100 cc.		Vol- ume.	Ca per 100 cc.		
				Mean.			Mean.	
	days	cc.	mg.	mg.	cc.	mg.	mg.	
1	0	1	13.5		1	6.9		Female, 4.6 kilos. Day of operation.
		1	12.2	12.8	1	6.6	6.8	Definite tetany.
	2	1	8.1		1	4.9		
		—	—	8.1	1	5.0	5.0	
	4	2	5.7		2	3.9		General tetany and hyperpnea.
		—	5.7	5.7	1	4.0	3.9	
2	0	2	11.4		1	7.7		Male, 11.5 kilos. Day of operation.
		1	11.5	11.4	—	—	7.7	Extreme tetany.
	1	1	6.9		1	5.4		
		—	—	6.9	1	5.8	5.6	
3	0	2	13.0		2	8.0		Male, 8.5 kilos. Day of operation.
		2	11.9	12.5	2	7.5	7.8	No tetany.
	2	2	9.2		2	6.0		
		2	8.6	8.9	2	6.0	6.0	
	5	2	8.3		2	5.2		Slight tetany of hind legs.
		2	8.1	8.2	1	5.7	5.4	
4	0	2	12.4		2	6.8		Female, 6.5 kilos. Day of operation.
		2	11.7	12.1	1	6.4	6.6	No tetany.
	7	2	7.6		2	5.1		
		2	7.6	7.6	1	5.7	5.4	
	10	2	5.2		1	4.7		" "
		2	5.6	5.4	—	—	4.7	
	11	2	6.4		2	4.9		Well marked tetany.
		2	6.3	6.4	2	5.3	5.1	
	14	2	5.2		2	4.8		Definite generalized tetany.
		2	5.0	5.1	—	—	4.8	After samples were taken, 12 cc. 3 per cent CaCl ₂ were injected subcutaneously. This was repeated next day.
	17	2	4.6		1	4.7		Acute tetany.
		2	4.7	4.7	1	4.7	4.7	
5	0	2	10.8		1	5.6		Male, 10.5 kilos. Day of operation.
		1	10.3	10.6	—	—	5.6	Distinct tetany. After samples taken, 5 cc. CaCl ₂ given subcutaneously.
	2	2	7.2		1	5.5		Fed milk.
		2	7.2	7.2	1	5.2	5.4	Definite tetany. No milk or Ca given.
	3	2	9.5		2	4.3		Extreme tetany.
		2	9.5	9.5	1	4.3	4.3	
	4	2	8.7	8.7	2	4.8	4.8	

The results for the first series of operated dogs are shown in Table II.

These results show the usual fall in serum calcium with the onset of tetany. But instead of the cerebrospinal fluid calcium showing a parallel fall and tending to disappear, *it is but slightly affected*, and a lowering of only 1 or 2 mg. of calcium in that fluid accompanies the onset of tetany symptoms. If the calcium in this fluid represents the diffusible calcium of the plasma, then this can be only slightly affected, and the main effect of removal of parathyroids on the blood calcium *appears to be* the complete, or almost complete, disappearance of the non-diffusible portion.

It is, of course, possible that instead of the disappearance of this portion of the blood calcium, its combination has been altered in such a way as to prevent its precipitation by oxalate after clotting of the blood. It seemed desirable to seek a method which would permit estimation of calcium after incineration in such small amounts of blood and cerebrospinal fluid as can conveniently be obtained in these experiments, and which could be applied to whole blood also.

Dienes has recently published a gravimetric method of calcium estimation (7), but this appeared much too complicated for our purpose.

At this juncture also Alport (8) published a paper "On the accurate estimation of calcium in whole blood," in which 8 cc. of blood are required for each determination, and after incineration the treatment is somewhat complex. We have modified Alport's procedure to suit our needs, and the modification appears to give satisfactory results with whole blood, serum, plasma, and cerebrospinal fluid.

In working with this method we have taken advantage of the fact, which does not appear to have been previously observed, that addition of a saturated solution of ammonium carbonate to the extent of 5 per cent (or more) to whole blood prevents clotting, though at once producing laking. Although some calcium carbonate is precipitated, on thoroughly shaking this "carbonated" blood, 1 or 2 cc. samples can be accurately pipetted off for analysis.

Modification of Alport's Method of Estimating Blood Calcium.

1 or 2 cc. of "carbonated" blood, or citrated or oxalated plasma, or serum, or cerebrospinal fluid, are accurately pipetted into a small platinum crucible, evaporated to dryness on the water bath, and then incinerated carefully over a low flame, at first heating the sides of the crucible, and slowly extending the flame downwards. The incineration is completed with a stronger flame, heating being continued till all carbon is completely oxidized. The ash is dissolved in 5 cc. of water containing 5 drops of concentrated hydrochloric acid. (If any creeping has taken place over the side of the crucible, this material is washed off with the solution into a small beaker, and the solution then poured back, with washing, into the crucible.) The solution is evaporated to dryness on the water bath, and the residue treated with 8 cc. of water containing 6 drops of glacial acetic acid, and 0.5 gm. of sodium acetate, and the solution evaporated to one-half or one-third on the water bath. It is then filtered through a small filter paper into a 15 cc. centrifuge tube, and the crucible washed three or four times with 1 or 2 cc. of water (to which a drop of glacial acetic acid is added). The washings are poured through the same filter paper into a porcelain dish, evaporated to 1 cc., and this added to the centrifuge tube; an additional cc. of water is used to wash the dish, and added to the tube. 1 cc. of concentrated ammonium oxalate is then added, and the subsequent procedure is that of the Tisdall modification of the Kramer-Tisdall method (5). With blood serum, oxalated or citrated plasma, and cerebrospinal fluid, the method gives no difficulty. With whole blood the ashing requires more care. (The filter paper we have used was Schleicher and Schüll, No. 590, 5.5 cm., HCl-HF-washed; blank tests showed no detectable calcium.)

Table III shows a comparison of the direct Tisdall procedure and this incineration method with normal serum and cerebrospinal fluid, and also some values for whole blood. All the whole blood samples were drawn into small bottles, graduated with a mark at the foot of the neck, and containing a definite amount of saturated ammonium carbonate solution. The blood was run in with shaking nearly to the mark, more ammonium carbonate run in to the mark from a graduated pipette, and from the known volumes of bottle and carbonate the necessary factor obtained.

The agreement for serum calcium and cerebrospinal fluid calcium adds additional proof that the Tisdall method estimates the whole of the calcium present in these normal fluids, and demonstrates that reasonable reliance can be placed on the incineration method that we have adopted. The figures for the whole blood of thirteen dogs, extremes 5.0 to 7.0, average 6.2 mg.

per 100 cc., are very similar to those obtained for human blood by Alport (extremes 5.4 to 6.9; average 5.8 mg.) and by Kramer and Tisdall (9) (extremes 5.3 to 6.7; average 5.8 mg.). The

TABLE III.

Sample No.	Source.	Kramer-Tisdall method.		Incineration method.	
		Volume.	Ca per 100 cc.	Volume.	Ca per 100 cc.
		cc.	mg.	cc.	mg.
1	Dog's blood serum.	2	10.8	2	10.4
		2	10.9	—	—
2	" " "	2	9.9	2	10.4
		2	10.5	—	—
3	" " "	1	10.9	1	10.0
4	" " "	2	8.6	2	9.6
		2	8.6	2	8.6
5	Human " "	2	10.1	2	9.9
6	" " "	2	9.6	2	9.7
7	Dog's cerebrospinal fluid.	1	4.9	1	5.2
8	" " "	1	6.3	1	6.4
9	Dog's whole blood.	—	—	2	5.4
				2	5.2
10	" " "	—	—	2	5.8
				2	5.8
11	" " "	—	—	2	4.9
				2	5.1
12	" " "	—	—	2	6.7
				2	6.7
13	" " "	—	—	2	6.2
14	" " "	—	—	2	6.2
15	" " "	—	—	2	5.0
16	" " "	—	—	2	6.5
17	" " "	—	—	2	6.1
18	" " "	—	—	2	6.9
19	" " "	—	—	2	6.6
20	" " "	—	—	2	6.7
21	" " "	—	—	2	7.0

actual variations are due largely to variations in the plasma volume of the bloods examined.

Accordingly we carried out a second series of experiments with parathyroidectomized dogs, to ascertain whether, in such ani-

imals, the Kramer-Tisdall method still gives the total amount of calcium present. The results are shown in Table IV, which includes also the figures for the later experimental animals.

These results are in good agreement with the first series. Although, with animals in acute tetany, both serum and cerebrospinal fluid calcium tend to approach the same low level, appearing to indicate that non-diffusible calcium may totally disappear, yet the decrease in diffusible calcium, indicated by the cerebrospinal fluid figures, is relatively slight. *The whole blood calcium decreases, but not in the same proportion as the serum calcium.*

The few figures for incinerated cerebrospinal fluid after parathyroidectomy show close agreement with those obtained by direct precipitation. In certain of those for serum the incinerated figure is slightly higher; this is probably due to experimental error.

In one case (normal serum from Dog 8) the figure obtained by the direct method is much lower than that obtained after incineration, and obviously does not represent the correct value. This may have been due to an error of determination, or may have been connected with the fact that this sample clotted very badly.

Believing that normal plasma contains its calcium in two forms, one diffusible (calcium ions and unionized inorganic calcium salts), and the other non-diffusible, presumably an organic calcium compound, we thought it possible that, although after clotting such an organic calcium compound might readily dissociate during the treatment with oxalate (and this is obviously the case from the agreement between the figures with and without incineration), yet with unclotted oxalated blood some difference of combination might exist, and plasma, removed by rapid centrifuging, might contain some calcium slowly dissociating, and slowly being precipitated by the excess of oxalate present. Indeed Lyman (10) states that he could not completely precipitate the calcium from beef and cat plasmas (Lyman speaks of "sera") by addition of excess of powdered potassium oxalate to the whole blood: "A considerable amount of calcium . . . escaped precipitation as oxalate."

We have accordingly oxalated normal dog's blood, centrifuged rapidly, and tested the plasma. Additional oxalate was added

TABLE IV.
Second Series of Experiments with Parathyroidectomized Dogs.

Dog No.	Time since operation.	Serum.				Cerebrospinal fluid.				Whole blood.		Remarks.
		Kramer-Tisdall method.		Incineration method.		Kramer-Tisdall method.		Incineration method.		Vol-ume.	Ca	
		Vol-ume.	Ca	Vol-ume.	Ca	Vol-ume.	Ca	Vol-ume.	Ca			
										cc.	mg.	
6	0	1	10.9	2	10.0	1	4.9	1	5.2	—	—	Male, 6 kilos. Day of operation. Fed milk next day. Definite but not acute tetany.
	2	1	5.6	1	5.6	1	4.6	1	4.6	—	—	
7	0	2	8.6	2	9.6	1	6.3	1	6.4	2	5.8	Female, 5 kilos. Day of operation. Developed marked tetany on this day.
	4	2	8.6	2	8.6	1	4.2	—	—	2	5.8	
	5	2	3.6	2	4.2	1	4.8	1	4.9	2	4.1	Marked general tetany. Blood from ventricle.
		2	3.6	2	3.7	1	4.8	1	4.9	2	4.0	
8	0	2	5.4	2	5.3	1	4.8	1	4.9	2	4.2	Female, 5.5 kilos. Day of operation. Marked tetany. 10 cc. 3 per cent CaCl ₂ given after samples taken. Extreme tetany.
	2	2	5.6	—	—	—	—	—	—	2	4.3	
	3	2	4.7	2	5.1	1	4.7	—	—	2	3.4	
	4	2	4.7	1	6.0	—	—	—	—	2	3.3	
	4	2	4.6	2	5.6	1	4.7	—	—	2	3.3	
		—	—	2	4.2	1	4.7	—	—	—	—	“ “ and exhaustion.

9	0*	2	10.4	—	—	2	5.3	—	—	1	6.2	Male, 14 kilos. Dec. 17.
	0	2	10.8	—	—	2	5.6	—	—	1	5.7	Dec. 22. Day of operation. Tetany de-veloped next day. Milk was fed and CaCl ₂ injected twice daily. Treatment alleviated the continuous slight tetany. Complete tetany. Treatment continued. Fed milk and powdered bones. Slight tetany. Ca withheld.
		2	9.9	—	—	2	5.5	—	—	—	—	Extreme tetany.
		2	10.1	—	—	2	5.5	—	—	—	—	Male, 13 kilos. Jan. 9.
10	9	2	3.8	—	—	2	3.8	—	—	2	4.5	Jan. 12. Day of operation. Slight tetany on 5th day. Ca on 5th and 6th days. General tetany. Ca given later and next day. Extreme tetany and exhaustion.
		2	3.6	—	—	2	3.7	—	—	2	6.9	Male, 18 kilos. Day of operation.
	14	2	4.2	—	—	2	4.1	—	—	2	6.1	Moderate tetany. Ca given on 6th and 7th days.
		2	4.3	—	—	2	4.1	—	—	—	—	Acute tetany. Ca given later and on suc-ceeding days.
11	16	2	3.8	—	—	2	3.5	—	—	2	5.0	Extreme tetany.
		2	3.9	—	—	2	3.5	—	—	2	—	Extreme tetany.
	0*	2	11.2	—	—	2	5.5	—	—	2	6.6	Male, 13 kilos. Jan. 9.
	0	2	11.4	—	—	2	5.6	—	—	2	4.4	Jan. 12. Day of operation. Slight tetany on 5th day. Ca on 5th and 6th days. General tetany. Ca given later and next day. Extreme tetany and exhaustion.
	7	2	10.0	—	—	2	4.8	—	—	2	4.0	Male, 18 kilos. Day of operation.
		2	5.2	—	—	2	4.6	—	—	2	6.7	Moderate tetany. Ca given on 6th and 7th days.
	10	2	4.0	—	—	2	4.1	—	—	2	—	Acute tetany. Ca given later and on suc-ceeding days.
		2	3.8	—	—	2	3.8	—	—	2	5.0	Extreme tetany.
	0	2	12.2	—	—	2	6.1	—	—	2	—	Male, 18 kilos. Day of operation.
		2	12.4	—	—	2	5.0	—	—	2	6.1	Moderate tetany. Ca given on 6th and 7th days.
	5	2	7.0	—	—	2	5.0	—	—	—	—	Acute tetany. Ca given later and on suc-ceeding days.
	8	2	6.3	—	—	2	(3.6)	—	—	2	—	Extreme tetany.
	14	2	6.3	—	—	2	(5.6)	—	—	2	—	Male, 18 kilos. Day of operation.
		2	3.6	—	—	2	4.5	—	—	2	6.1	Moderate tetany. Ca given on 6th and 7th days.
		2	3.8	—	—	2	4.5	—	—	2	—	Acute tetany. Ca given later and on suc-ceeding days.
		2	3.6	—	—	2	4.5	—	—	2	5.0	Extreme tetany.

* Several days prior to operation.

TABLE IV—*Concluded.*

Dog No.	Time since operation.	Serum.				Cerebrospinal fluid.				Whole blood.		Remarks.
		Kramer-Tisdall method.		Incineration method.		Kramer-Tisdall method.		Incineration method.		Vol-ume.	Ca	
		Vol-ume.	Ca	Vol-ume.	Ca	Vol-ume.	Ca	Vol-ume.	Ca			
		cc.	mg.	cc.	mg.	cc.	mg.	cc.	mg.	cc.	mg.	
12	0	2	11.3	—	—	2	6.2	—	—	2	7.0	Male, 12.5 kilos. Day of operation. De-veloped tetany on succeeding day. Ca injected. Moderate tetany with marked hyperpnea. Ca on 3rd and 4th days. Acute tetany.
		2	11.1	—	—	2	6.2	—	—	2	4.6	
	2	2	5.6	—	—	2	4.7	—	—	2	5.3	
		2	5.0	—	—	2	5.2	—	—	2	5.3	
	5	2	5.0	—	—	2	5.2	—	—	2	5.3	
		2	5.0	—	—	2	5.2	—	—	2	5.3	

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in the usual manner after varying periods and the calcium estimated directly by Tisdall's method; samples were also incinerated, and the calcium was then estimated. The plasma was thoroughly shaken before sampling, as we considered that with

TABLE V.
Calcium in Oxalated Blood Plasma.

Dog No.	Condition.	Kramer-Tisdall method.			Incineration method.	
		Time.	Volume.	Ca	Volume.	Ca
		hrs.	cc.	mg.	cc.	mg.
D	Normal.	Soon.	2	2.5	—	—
E	"	"	2	2.6	—	—
F	"	"	2	3.3	—	—
G	"	1.75	2	0.5	2	1.6
L	"	4.25	2	1.6	—	—
M	"	2	2	0.5	2	1.8
	"	2	2	0.6	—	—
9	"	—	—	—	2	2.2
	"	—	—	—	2	3.0
	"	Soon.	2	1.3	2	3.3
	9 days after; complete tetany.	2	2	0.6	2	2.1
	14 " " slight "	1.5	2	1.5	2	1.9
	"	1.5	2	1.9	—	—
	16 " " extreme "	1.75	2	2.1	—	—
	"	5	2	1.5	—	—
10	Normal.	Soon.	2	1.8	2	2.4
	7 days after; general tetany.	—	—	—	2	3.4
	10 " " extreme "	—	—	—	2	2.3
11	Normal, Jan. 23.	—	—	—	2	3.9
	" " 28.	—	—	—	2	4.0
	5 days after; moderate tetany.	—	—	—	2	4.3
	14 " " acute "	—	—	—	2	2.1

slow dissociation in the presence of the excess of oxalate initially added to prevent clotting some calcium oxalate would slowly separate. We also carried out a similar series of determinations with the oxalated plasma from several dogs after parathyroid-

tomy. The results are given in Table V. The times stated are those elapsing from the obtaining of the blood sample till completion of the centrifuging of the oxalate precipitate.

The results with normal animals seemed to support our hypothesis of a slow dissociation of an organic calcium compound after removal of calcium ions. That figures only between 2 and 4 mg. were obtained might be attributed to the continued slow dissociation after oxalation, and removal of some of this fraction as oxalate when centrifuging to obtain the plasma.

But the results with the operated dogs in tetany appear to invalidate this conclusion. The remarkable agreement between the final values of serum and cerebrospinal fluid for six operated dogs (Nos. 4, 7, 8, 9, 10, and 12) seems to us entirely to warrant our assumption that cerebrospinal fluid calcium represents the diffusible calcium of the plasma. Hence, though, finally, non-diffusible calcium seems to have entirely disappeared from the serum of Dogs 9, 10, and 11, yet *the oxalated plasma contained calcium of the same order as does the oxalated plasma of normal animals.*

We must now consider also the fact that Dogs 7, 9, 10, 11, and 12 showed at certain stages after parathyroidectomy calcium in the whole blood equal to or greater than that present in the corresponding serum.

Kramer and Tisdall² have summarized the evidence for the presence of calcium in blood corpuscles, and have drawn the conclusion, which seems almost certainly correct, that *the corpuscles contain no calcium*; this is supported by the results of Rona, Petow, and Wittkower (11), indicating that, under conditions existing in blood, hemoglobin shows no tendency to unite with calcium. Yet the results with whole blood from parathyroidectomized dogs suggest that the concentration throughout the blood is at least as great as that in the serum, and, therefore, as that in the plasma, since according to Howland and Kramer (12) there is no appreciable difference between the calcium content of serum and plasma.

It does not appear probable that as a result of parathyroidectomy some part of the blood calcium is transferred to the cor-

² Kramer and Tisdall (9), p. 245.

puscles; this would not explain the presence of slowly dissociating calcium in the oxalated plasma of the operated dogs.

We were therefore forced to consider the possibility that there is present in normal blood plasma a specific organic calcium compound, which before clotting only slowly reacts with oxalate, but which is changed during clotting so that afterwards all the calcium is completely precipitated by ammonium oxalate within half an hour, whereas in the acute tetany following parathyroidectomy the organic calcium is held entirely in the clot.

If this explanation is correct, then after parathyroidectomy and in acute tetany, plasma calcium should be greater than serum calcium by at least the 2 to 4 mg. per 100 cc. present in the oxalated plasma, while normally plasma and serum calcium should be equal, as Howland and Kramer (12) and Wang and Felsher (13) have concluded.

It should be possible to test this explanation by comparing the calcium in citrated plasma and in serum. Unfortunately the addition of concentrated (or solid) sodium citrate in sufficient amount to prevent clotting results in corpuscular shrinkage, and to a greater extent than is usually taken into account. This causes such a dilution of the plasma as to necessitate a definite correction. Further, hematocrit determinations as usually carried out give too high figures for corpuscular volume and no certain correction for plasma volume changes can be ascertained by such means. We have determined on normal dog's blood the approximate effect of citrate, using a saturated solution of sodium citrate rendered just acid to litmus by addition of saturated citric acid. Addition of 1 volume to 100 volumes of blood gave 58.8 volumes of corpuscles, of 2 volumes, 56.3, of 4 volumes, 50.5, and of 10.6 volumes, 45.3; these results give a fairly satisfactory curve indicating that the true corpuscular volume determined under the same centrifuging conditions would lie between 60 and 61 volumes. Accepting the lower figure, the observed plasma volume after addition of 1 volume of citrate would be $41.2 + 1$, *i.e.* 42.2, and the true volume 40. Any determination on the constituents of such a plasma therefore requires a correction given by the factor $\frac{42.2}{40} = 1.05$. This correction is somewhat too large, since the corpuscular volumes as determined

are too great, but the size of the correction indicates that it is not negligible, even with minimum citrate addition.

(The citrate curve, as determined, is complex, since not only is there corpuscular shrinkage, but the flattened corpuscles permit closer packing under the same centrifuging conditions. This prevents any simple mathematical extrapolation from citrate calcium figures with different volumes of citrate.)

A second important error may arise from addition of insufficient oxalate for complete precipitation of calcium, since there will result a balance between calcium citrate and oxalate. Our figures in Table VI, comparing incinerated citrated plasma and citrated plasma precipitated by excess of oxalate and allowed to remain 18 or more hours before centrifuging off the calcium oxalate, indicate the importance of this error. Clark³ has drawn attention to the time factor in this precipitation. A third error consists in the use of alkaline citrate (*cf.* Kramer, Tisdall, and Howland (14)); the alkalinity may cause definite precipitation of calcium phosphate, which, in whole or part, will be removed during centrifuging for plasma, so that too low results are obtained. The figures in Table VI in brackets were for plasmas obtained by such alkaline citrate and tend to be low. We have used in all the other determinations a solution made just acid to litmus by the addition of 14 cc. of saturated citric acid to 100 cc. of saturated sodium citrate. All three errors lead to too low results.

We have attempted to obtain approximate corrections for our citrate results by centrifuging in hematocrit tubes always under comparable conditions (20 minutes at about 2,000 revolutions per minute) and determining the corpuscular volume. The factor was then obtained from the observed plasma volume divided by that figure minus the volume of citrate added (*i.e.*, we make the reasonable assumption that, since the corpuscles shrink, this citrate is added to the plasma only). For small additions of citrate this factor is too large, since the plasma held between the corpuscles, X volumes, has to be added to the observed plasma, P volumes, and corrected plasma, P_c volumes,

³ Clark (6), p. 505.

TABLE VI.

Comparison of Serum Calcium and Citrated Plasma Calcium.

Dog No.	Date.	Serum Ca.	Volume of citrate added.	Observed plasma volume.	Factor.	Plasma Ca determined by incineration.	Corrected plasma Ca.	Plasma Ca determined by addition of oxalate.	Condition of animal.
		mg.	per cent	per cent		mg.	mg.	mg.	
J	Jan. 12	9.9	10.7	65.0	1.31	(6.8)	(8.9)	—	Normal.
K	" 14	11.6	8.1	46.0	1.18	(8.0)	(9.4)	—	"
L	" 21	11.1	2.1	47.0	1.04	11.0	11.4	—	"
			12.2	54.1	1.23	9.5	11.7	2.7	
M	" 29	10.3	1.1	42.3	1.03	14.1	14.5	—	"
			11.2	52.2	1.21	10.5	12.7	—	
10	" 12	10.0	12.2	51.0	1.24	(8.1)	(10.0)	—	"
	" 19	5.2	2.0	53.6	1.04	8.8	9.2	—	General tetany.
			10.7	58.3	1.19	7.6	9.0	—	
	" 22	3.9	1.9	62.2	1.03	7.8	8.0	4.2	Extreme tetany.
			10.9	68.7	1.16	7.0	8.1	2.3	
11	" 23	12.6	2.1	36.4	1.06	12.3	13.0	—	Normal.
			10.7	46.9	1.23	9.5	11.7	—	
	" 28	12.3	0.9	32.4	1.03	11.4	11.7	—	"
			1.9	33.1	1.06	13.7	14.5	—	
	Feb. 2	7.0	1.0	42.2	1.02	9.8	10.0	8.3	Moderate tetany.
			2.0	42.2	1.05	10.3	10.8	6.6	
			10.8	48.4	1.22	9.9	12.1	—	
	" 5	6.3	1.0	47.8	1.02	8.5	8.7	7.1	Acute tetany.
			2.0	50.7	1.04	9.0	9.4	6.9	
			11.2	53.6	1.21	7.3	8.8	3.2	
	" 11	3.7	1.0	54.9	1.02	7.2	7.3	4.1	" "
			2.0	57.9	1.03	7.3	7.5	4.2	
			10.8	61.8	1.17	—	—	0.9	
12	" 4	11.2	0.9	30.8	1.03	14.0	14.4	—	Normal.
			10.4	47.4	1.22	11.6	14.2	—	
	" 6	5.6	1.0	34.7	1.03	9.3	9.6	—	Marked tetany.
	" 9	5.0	1.0	52.5	1.02	7.6	7.8	6.7	Acute tetany.
			2.2	51.8	1.04	7.6	7.9	5.1	
			(11.8)	—	—	—	—	1.7	

so that

$$\frac{P}{P_o} > \frac{(P + X)}{(P_o + X)}$$

The true value, therefore, should lie between the observed value and the corrected figure. With addition of larger amounts

TABLE VII.

Dog No.	Condition.	Cerebrospinal fluid Ca. <i>mg.</i>	Serum Ca. <i>mg.</i>	Citratd plasma Ca.		Oxalated plasma Ca. <i>mg.</i>	Whole blood Ca. <i>mg.</i>	$\frac{\text{Cerebrospinal fluid Ca.}}{\text{Citratd plasma Ca.}}$
				Extremes. <i>mg.</i>	Approximate mean. <i>mg.</i>			
10	Normal.	4.8	10.0	(10.0)	10.0	2.4	6.6	0.48
	After 7 days; general tetany.	4.4	5.2	8.8- 9.2	9.0	3.4	4.4	0.49
	After 10 days ; extreme tetany.	3.8	3.9	7.8- 8.1	8.0	2.3	4.0	0.47
11	Normal (Jan. 23).	—	12.6	12.3-13.0	12.7	3.9	—	—
	" (" 28).	6.1	12.3	11.4-14.5	13.0	4.0	6.7	0.47
	After 5 days; moderate tetany.	5.0	7.0	9.8-10.0	9.9	4.3	6.1	0.51
	After 8 days; acute tetany.	(4.6)	6.3	8.5- 9.4	8.9	—	—	(0.52)
	After 14 days; acute tetany.	4.5	3.7	7.2- 7.3	7.3	2.1	5.0	0.51*
12	Normal.	6.2	11.2	14.0-14.4	14.2	1.9	7.0	0.44
	After 2 days; acute tetany.	4.7	5.6	9.3- 9.6	9.5	—	4.6	0.49
	After 5 days; acute tetany.	5.2	5.0	7.6- 7.8	7.7	—	5.3	0.68

* The serum calcium is definitely lower than that of the cerebrospinal fluid, indicating that readjustment between blood and cerebrospinal fluid is not complete. The serum calcium figure has been used for the ratio.

of citrate (of the order of 10 volumes) through corpuscular shrinkage and closer packing the actual plasma as read, less the citrate added, should approximate more closely to the true plasma volume of the original blood, and may even be greater, so that theoretically

the corrected figures for such plasmas should be less in excess of the true value, and might be definitely smaller. The much greater correction necessary exaggerates the error of determination so that these figures can only be used as confirming the order of the others.

TABLE VIII.

Relative Corpuscular Volumes of Dog's Blood before and after Parathyroidectomy.

Dog No.	Condition and time from operation (days).	Measured corpuscular volume.
		<i>per cent</i>
9	Normal (Dec. 17).	69
	" (" 22).	67.5
	Tetany; 9 days.	55
	" 14 "	48.5
	" 16 "	38.5
10	Normal (Jan. 9).	62.5
	" (" 12).	55.5
	Tetany; 7 days.	55.5
	" 10 "	44
11	Normal (Jan. 23).	72
	" (" 28).	70.5
	Tetany; 5 days.	65
	" 8 "	58.5
	" 14 "	50.9
12	Normal.	71.2
	Tetany; 2 days.	73.5
	" 5 "	54.3

Our results with citrated plasmas are shown in Table VI. The amounts are in mg. per 100 cc.

The results for normal plasma show usually a moderate agreement with serum values. In two cases the plasma value is higher. Evidently further examination of plasmas obtained without the use of citrate or similar agents is necessary. If such higher values as we have found are correct it would appear that in normal dogs the blood plasma calcium can occasionally exceed the usual limits, and when such blood clots some part of the calcium is held in the clot.

The results for dogs in acute tetany show that their plasma always contains 3 or more mg. of calcium per 100 cc. *which are not present in the serum*, and must, therefore, be held in the clot. The figures for oxalate plasma are sufficiently close, when loss from continued slight dissociation is remembered.

TABLE IX.

Time of Onset and Occurrence of Hyperpnea in Parathyroidectomized Dogs.

Dog No.	Onset of tetany after operation.	Hyperpnea as prominent early symptom.	Remarks.
	<i>days</i>		
1	2	+	Marked tetany; later hyperpnea.
2	1	+++	Hyperpnea followed by marked twitchings.
3	5	+	Moderate tetany; conjunctival and nasal irritation.
4	11	—	Slight tetany, rapidly becoming general.
5	2	—	Complete general tetany; diaphragm spasm.
6	2	—	Milk given after operation; slight tetany, rapidly becoming general.
7	2	—	Milk given after operation; slight tetany, slowly becoming general.
N	1	++	Attacks of hyperpnea and tetany rapidly developed. Diaphragm and abdominal contractions synchronized with heart-beat.
8	2	—	Muscular cloni.
9	1	++	Tetany developed first; then hyperpnea, the tetany rapidly became intense.
10	3	—	Slight tetany, slowly becoming general.
L	5	—	Fed milk and given calcium lactate injection after operation. Slight tetany.
11	5	—	Milk 1 day after operation. Moderate tetany, hyperpnea developing later.
12	1	+++	Hyperpnea the initial symptom.

The essential figures for the last few operated animals are combined in Table VII. The amounts are all in mg. per 100 cc.

We have also attempted to determine directly the diffusible calcium from the "carbonated" blood by direct precipitation with oxalate. After addition of oxalate 3 or 4 hours are required before constant figures are obtained. The results for seven

normal dogs varied between 34 and 52 per cent of the total calcium found by incineration of the same blood, averaging 42 per cent, a smaller figure than that determined by the ratio between cerebrospinal fluid and serum calcium (53 per cent). The results are illusory, since under the conditions employed some of the organic calcium would be precipitated, leading to too high a figure, while the markedly increased alkalinity would result in precipitation of calcium phosphate, which would not be estimated by permanganate so that too small a figure would result.

The series of hematocrit determinations on oxalated blood, though yielding too high figures for corpuscular volume, are comparable, since they were carried out under the same conditions. The results are shown in Table VIII, the corpuscular volumes having been corrected for the oxalate added.

Table IX summarizes the observed time of onset of tetany. It would appear that where hyperpnea is the first symptom observed tetany tends to ensue earlier; in all cases the hyperpnea, whether preceding or induced by the tetany, intensifies the nervous manifestations. This is to be expected from the observations of Collip and Backus (15) and of Grant and Goldman (16) on the tetany induced by forced breathing.

DISCUSSION.

Diffusible Calcium of Normal Blood.

In considering the partition of calcium in the blood and similar fluids the terms "ionized" and "ionic" calcium, which have been used by Vines (17) and by Salvesen, are misleading, and the term "ionizable calcium" would be still more inaccurate, since apparently the organic calcium compound of the blood can ionize. We prefer to speak of "diffusible calcium" as referring to that fraction which includes calcium ions and unionized inorganic calcium compounds and which can diffuse through a normal functioning animal membrane.

A number of attempts have been made to measure the diffusible calcium of the blood, using collodion and similar membranes. Rona and Takahashi (18) found for the sera of the horse, pig, and cow, that from 65 to 75 per cent of the serum calcium is dialyzable. In a subsequent paper (19) they showed that slightly

ionized compounds of the type of CaHPO_4 will diffuse. They considered that, on account of the but slight traces of fatty acids present in blood calcium, soaps are unlikely, and a calcium-protein complex is more probable as the form of combination of the undiffusible calcium.

Von Meysenbug, Pappenheimer, Zucker, and Murray (20) obtained similar results (60 to 70 per cent for normal men and dogs) and showed that these are not affected by slight changes of pH. Cruickshank (21) obtained slightly lower figures with dogs (55 to 70 per cent), using a special parlodion membrane, presumed to give maximum permeability to crystalloids, and to be impermeable to colloids.

R. F. Loeb (22) finds that serum calcium is completely diffusible through collodion membranes into 0.8 per cent sodium chloride at pH 7.4, while 55 to 75 per cent will diffuse against distilled water at this pH.

(We have carried out a few experiments, in which parlodion sacs containing exactly 3 cc. of serum were immersed in 9 cc. of distilled water to equal levels. After 24 hours in stoppered containers, the concentration of calcium in the external liquid was, within the limit of error, exactly one-fourth of that in the original serum, so that complete calcium equilibrium resulted. These results do not confirm those of Loeb.)

Obviously Loeb's method is less suitable than compensation-dialysis procedures, since upset of the equilibrium between organic calcium and calcium ions should at once lead to some dissociation of the organic compound. The same criticism may also apply to Cushny's procedure of filtration under slight pressure (23); his results are, however, similar (62 and 70 per cent in the two measurements made). With a similar ultrafiltration method Neuhausen and Pincus (24) found that from 50 to 70 per cent of pig's serum calcium is diffusible, and H. and C. Tschimber (25), 50 to 60 per cent (human pathological sera).

All dialysis methods are open to the objection that, under the experimental conditions employed, varying permeability exists. We believe we are right in concluding that the capillary wall and endothelial membranes of the choroid plexuses are a more perfect dialyzer than any used in the experimental methods referred to, and also than in the abnormal conditions in nephritis which

permit the production of edematous fluid through increased pressure (and are, therefore, more comparable with Cushny's method). Our results, however, apply to *plasma* dialysis; most of those referred to above were obtained with *serum*.

Vines (17), by an ingenious but less accurate biological method, obtained some results from which it may be concluded that the diffusible calcium (his "ionic" calcium) is about 63 per cent of the *plasma* calcium.

Assuming that in most cases the values of serum and plasma calcium are equal, our figures in Table I show that the diffusible calcium of the plasma, dialyzed into the cerebrospinal fluid varied in twenty-two determinations on twenty dogs between 42 and 73 per cent. Fifteen of these determinations were within the limits of 48 and 56 per cent, and the average for all was 53 per cent. The figures for cerebrospinal fluid calcium in seventeen determinations did not exceed the limits of 4.8 and 6.3 mg. per 100 cc.

The ratio shows much greater constancy than the figures published by Critchley and O'Flynn (4) for neuropathic human cases. A number of their results for serum seem unusually high (17 to 19 mg. per 100 cc.), but unfortunately they give no particulars as to the type of case to which these high figures refer. With the use of accurate analytical methods other figures of this order have only been recorded by Coates and Raiment (26) for gouty patients (18.9 mg. average).

Barrio (27), working almost entirely on material from cases undergoing treatment for syphilis, obtained much more constant results, his extremes being for fourteen cases 45 to 57 per cent, and the average 49 per cent.

We conclude from our results that *the average true diffusible calcium of blood plasma from normal dogs is 53 per cent of the serum calcium value*. We shall give reasons for believing that the diffusible calcium of the *serum* may well be greater than that of the *plasma*.

Diffusible Calcium of Abnormal Blood.

Kramer, Tisdall, and Howland (14) have emphasized that the **only** conditions in which serum calcium is lowered are nephritis, tetany, and rickets (in which latent tetany may be presumed).

The only observations that appear to have been made in the diffusible calcium in nephritis are those of Salvesen and Linder already referred to, and their results really indicate a partition, through a perhaps damaged membrane, under the effect of increased pressure.

Few observations have been made on diffusible calcium in tetany. MacCallum and Vogel (28) appear to conclude that the percentage diffusible is not much altered. Von Meysenbug and McCann (29), using the compensation-dialysis method, found similar figures to those for normal serum in two cases of active rickets, and for four parathyroidectomized dogs (58 to 71 per cent). On the other hand, Cruickshank (21), using parlodion tubes and the compensation method, found in the tetany following parathyroid removal in seven dogs, that the diffusible calcium amounted to 94 per cent.

Our figures in Tables II and IV show that when marked tetany has developed after parathyroidectomy, the diffusible calcium of the *plasma*, as determined by the figures for cerebrospinal fluid, although decreasing in actual amount, yet gradually *increases to from 70 to 100 per cent of the serum calcium value.*

Calcium of Whole Blood.

As we have pointed out, our figures for dogs by a modification of Alport's method are in good agreement with those of Alport and of Kramer and Tisdall for normal human blood. Kramer and Tisdall have pointed out that the higher figures in such papers as those of Jones and Nye (30) are almost certainly due to errors in technique. Cruickshank's high figures for normal dog's blood (average 9.1 mg.) are presumably liable to the same criticism.

After parathyroidectomy the calcium of the whole blood definitely decreases; the decrease affects both the diffusible and non-diffusible calcium.

Calcium Content of Blood Plasma.

We have pointed out the various sources of error which must be considered if plasma calcium is to be estimated on citrated blood. It would seem doubtful if the published figures on citrated plasma have been corrected for such errors. They probably account for

the discrepancies observed by Wang and Felsher (13) between the plasma and serum calcium values. Cruickshank's results for whole blood are so obviously incorrect that no stress can be laid on the corresponding high values he obtained for citrated plasma.

Most of our figures support the conclusion usually reached that the calcium content of normal serum is equal to that of the corresponding plasma. In two cases, however, the plasma figures are definitely *higher*, and the difference appears to be too great to be attributable to experimental error. We hope to check such results with plasmas to which have been added no citrate or similar agents which disturb osmotic equilibria.

The figures for plasma calcium after parathyroidectomy, whether determined by incineration, or by direct precipitation (with minimally citrated bloods) are definitely higher than those for the corresponding serum.

They indicate that the blood plasma of parathyroidless animals contains a proportion of calcium which is not diffusible, and which does not pass into the serum on clotting. This conclusion is supported by the existence of slowly dissociating calcium in the oxalated plasma from such animals. Further, the figures afford an explanation of the fact that the whole blood from such animals gives calcium values equal to, or even slightly greater than, the corresponding serum values.

Constancy of Calcium in the Blood.

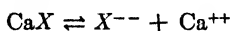
In normal dog's blood the calcium is remarkably constant, averaging about 6 mg. per 100 cc., and it is difficult to increase it perceptibly by feeding calcium. In the blood of other normal animals there is similar constancy and a similar amount. To what is this constancy due? Possible controlling factors are the reservoir of calcium salts in bone, and a kidney and intestinal "dam."

In the growing animal, depositing calcium salts in bone, the blood calcium is slightly higher than in adults (Meigs, Blatherwick, and Cary (31)), as is also apparently the cerebrospinal fluid calcium (Brock (32)), while in the pregnant woman, with an extra demand for calcium for the fetus, blood calcium is slightly lower (Widdows (33)). Howland and Kramer (12) have

pointed out that the constancy of calcium, inorganic phosphorus, and bicarbonate in the plasma of growing children undoubtedly determines the constancy of the inorganic composition of normal bone. If growing animals, with higher calcium in blood, deposit bone of constant composition on account of the constancy of blood calcium, and if the strain of bone formation tends to lower the blood calcium (as in pregnant women) then we can scarcely suppose that in adults, constantly excreting calcium, and therefore provided with excess, the constancy of blood calcium depends on a bone reservoir; rather the cessation of deposition of bone salts must be connected with the lowered calcium of the blood of the adult. In calcium starvation the reservoir of bone calcium is drawn upon.

If we admit that kidney epithelium possesses some damming action which holds back calcium to a constant value, yet we can scarcely believe that the great surface of the intestinal epithelium possesses the same property; more calcium is excreted through the intestines than through the kidneys. Nor can we agree with Salvesen that this vast intestinal threshold is under the control of a compound secreted by the parathyroid.

Our results indicate that there is present in normal blood plasma an organic calcium compound, which, when all calcium ions are removed, slowly dissociates. Such a compound is usually assumed to be a protein combination, but as we shall show, the organic radical must be specific, and not one of the ordinary plasma proteins. We shall term it X . Since Table V shows that this compound, CaX , slowly gives up its calcium in the absence of calcium ions, it may be assumed to dissociate slightly, and to be normally in equilibrium with calcium ions:



and

$$\frac{(X^{--}) \times (\text{Ca}^{++})}{(\text{CaX})} = k \text{ (a small constant)}$$

If under normal conditions the amount of X is constant, then, since normally excess of calcium reaches the blood stream from the diet, we can perhaps consider that the maximum amount of CaX will be formed, and this will hold a definite concentration of calcium ions in equilibrium in the plasma. Other interlocked

equilibria will be set up with bicarbonate and phosphate ions, etc., resulting in formation of definite amounts of unionized inorganic calcium compounds. In the presence of excess calcium ions the series of equilibria will ultimately depend on the organic compound X which can unite with calcium, and excess calcium will be excreted (will flow away).

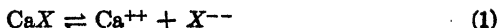
After parathyroidectomy the amount of X diminishes, and $\text{Ca}X$ also. Consequently a proportional part of the inorganic calcium should also disappear, since the inorganic calcium salts are diffusible. This actually takes place. The figures in the last column of Table VII, showing the ratio between the cerebrospinal fluid calcium (equal to the diffusible calcium of the plasma) and the citrated plasma calcium (total plasma calcium), are approximately constant (about 50 per cent) in two cases; in the third, with initial abnormally high plasma calcium, the organic calcium diminishes more rapidly.

If this theory be correct, then *the constancy of the blood calcium depends on an adequate supply of calcium from the diet, and on the presence in the blood of a specific organic compound, which unites with calcium, and only dissociates slightly.*

Collip has just claimed, apparently with reason, to have isolated a highly potent extract of the specific compound of the parathyroid. We have only had access to his note in the Canadian Medical Association Journal (34) in which he states that the "parathyroid hormone" produces "its beneficial effect" in relieving tetany in parathyroidectomized dogs "through an elevation of the level of blood calcium." If this compound is, or more probably controls the formation of the organic compound X , and its normal behaviour during clotting, then our results may be of assistance in furthering his work.

Forms of Combination of Calcium in Blood Plasma and Serum.

Our results indicate that on the average not more than 53 per cent of the *plasma* calcium is in diffusible form. The dialysis methods with *serum* indicate that at least 60 per cent of serum calcium is in diffusible form. The following series of interlocked equilibria probably represent the chief forms of calcium present in plasma.



The proteins in formula (4) are assumed to be the ordinary plasma proteins, and the amount of calcium taking part in the reaction of formula (4) is negligible. For, if calcium is capable of uniting with plasma proteins functioning as acids, the amount of Ca-protein so formed should bear a ratio to Na-protein of the same order as that of plasma calcium to plasma sodium (about 1:30). Rona, Haurowitz, and Petow (35) have shown by the rapid dialyzation method that serum sodium is completely diffusible at pH 7.8. Neuhausen and Pincus (24) and H. and C. Tschimber (25) have shown its complete, or almost complete, diffusibility by the ultrafiltration method, and Pincus and Kramer (36) obtained the same values for serum and cerebrospinal fluid sodium. It may therefore be concluded that plasma sodium only unites with plasma proteins to a very slight extent, and that the corresponding calcium compounds are negligible.

Vines (17) has shown that as blood clots the amount of calcium that will react with oxalate gradually increases, until it is 100 per cent precipitable. Yet this calcium is not all in inorganic combination, as he appears to conclude, since the dialysis experiments show that 40 per cent will not dialyze. Evidently the change is in the radical X, which we may assume provisionally changes to Y, where Y is also an organic radical of large molecular size. CaY reacts more easily with oxalate, so that calcium is completely precipitated in half an hour, whence it may be concluded that it dissociates to a greater extent, and

$$\frac{(Y^{--}) \times (\text{Ca}^{++})}{(\text{CaY})} = k' \text{ (where } k' > k \text{)}$$

The resultant increase in calcium ions would appear to explain the apparent fact that less plasma than serum calcium is diffusible.

Such explanations appear also to account for the exact agreement found in six cases of acute tetany between the serum calcium determined by direct precipitation with oxalate (the dif-

fusible calcium of the plasma) and cerebrospinal fluid calcium, since in this condition all the CaX has passed to the clot, and consequently there is no redistribution of calcium in the serum on clotting.

Evidently the relative stability of CaX in the presence of oxalate indicates that some of its properties can be ascertained by examination of oxalated plasma, and we hope to study this part of the problem further.

Cause of Tetany in Parathyroid Deficiency.

In this paper we do not propose to discuss the causation of tetany in forced breathing, nor in the various toxic conditions which may, or may not, produce the symptoms through production of parathyroid hypofunction. Neither do we propose to add to the academic discussion as to whether the immediate cause of tetany is calcium deficiency or sodium increase. Obviously a diminution of calcium content in the circulating blood will lead to a relative increase of the sodium content, so that a sodium-calcium balance will be disturbed.

We consider that after parathyroidectomy it has been amply demonstrated that one of the chief effects is a diminution of blood calcium, and since such a slight diminution is known to increase nerve excitability it can be justly concluded that this diminution is the immediate cause of the symptoms which develop.

The lowest figure for serum (and cerebrospinal fluid) calcium which we have observed in acute tetany is 3.7 mg., and the usual figure for serum calcium is between 4.5 and 6 mg. per 100 cc., in good agreement with the results of other observers.

We have shown that the main effects of parathyroidectomy on blood calcium are a diminution of total calcium, diffusible calcium, and non-diffusible calcium, and some change whereby the organic calcium passes into the blood clot. We have suggested that the amount of diffusible calcium present in the blood is, through a series of equilibria, determined by the amount of organic calcium, diminution of the latter resulting in a corresponding diminution of the inorganic calcium. *Tetany, therefore, is only an indirect result of parathyroidectomy, due to a decrease of some organic calcium compound causing through disturbed equi-*

bria a slight decrease in the inorganic calcium of the blood, which leads to the condition.

Parathyroid Control of Bone Growth.

This question is outside the scope of this paper, but the dependency of the height of blood calcium on efficient parathyroid function with the higher value in blood calcium during growth already referred to, strongly suggests that the parathyroid is concerned. This is supported by the recent observation of Doyle (37) that in rachitic chickens, in which latent tetany and calcium deficiency may be assumed, there is constant enlargement of the parathyroids. This may be compared with the hypertrophy of the thyroids which occurs in the initial stages of goiter through iodine deficiency.

Clotting of Blood.

Simpson and Rasmussen (38) were unable to show that removal of the parathyroids from dogs had any influence on blood clotting. We find that the blood from our animals in acute tetany after parathyroidectomy clots in normal time and clots well.

Since oxalation of blood prevents clotting, although the organic compound CaX only slowly dissociates, and is present for some hours in the unclotted blood, we cannot conclude with Vines (17) that clotting depends essentially on the organic calcium compound and does not require the presence of calcium ions, but must hold to the usual view that the prevention of clotting by oxalate (and similar agents) is due to removal of calcium ions. Since, during clotting, the organic calcium compound is apparently changed, it would appear, however, that this compound does play some essential part in clotting (as well as the calcium ions); to this extent we are in agreement with Vines. Obviously, also, the properties of this compound are affected by removal of the parathyroids, so that it would appear that the process of clotting in the blood of the parathyroidectomized animal is to some extent altered.

Treatment of Parathyroid Tetany.

* Experimental animals, whose parathyroids have been removed, can be kept free from tetany, as Salvesen has shown, by constant

administration of calcium salts, or increased milk diet (increased calcium in the diet). Under these circumstances their blood calcium seldom rises above 7 mg. per 100 cc. of serum.

If the theory that we have suggested as an explanation of the constancy of blood calcium be accepted, the lower maximum attained in these latent tetany animals can be understood. They are still deficient in the organic calcium compound, so that their serum calcium can only be maintained above the low level of 4 or 5 mg. per 100 cc. (and above the threshold at which tetany occurs) by constant administration of calcium; the excess can flow away so rapidly, and through such a large surface (the intestinal epithelium) that no marked increase is to be expected.

It follows that calcium administration in the clinical alleviation of tetany should be most effective if repeated small doses are given by mouth (five or six times a day). Larger doses will probably only be slightly absorbed.

(In Inouye's experiments in preventing tetany by administration of large amounts of lactose (39), apparently the lactose contained slight traces of calcium, the salt mixture contained more, and initial tetany was alleviated with calcium lactate. Our results indicate that only small continuous traces of calcium from the diet are necessary.)

It has, of course, been shown that administration of calcium chloride (40) or of ammonium chloride (41) produces an acidosis which is apparently effective in counteracting the alkalosis believed to be produced in tetany. It has been shown, also, that administration of mineral acid is effective in relieving the tetany condition in dogs for at least several days (42), but there are as yet no data to show that long continued treatment with a compound producing acidosis, other than calcium salts, can be safely employed in tetany. If calcium salts are used, then, from the point of view of treatment it is immaterial whether the beneficial effect is due to production of a continued acidosis, or to increase of calcium ions in blood (and tissues) above a dangerous minimum.

Effect of Thyroparathyroidectomy on the Red Blood Corpuscles.

Müller, Fleiner, Falta, and others (quoted by Barker (43)), have reported increased red cells in acute tetany, while according

to Janney (44) there is regularly a reduction of red cells in hypothyroidism. Cruickshank's figures⁴ for thyroparathyroidectomized dogs show marked changes in both directions.

Our hematocrit results, carried out on oxalated blood always containing the same amount of oxalate, and centrifuged under comparable conditions, show considerable variations, and always a slight drop after the dog had been kept in the laboratory on proper diet for several days, seeming to indicate that winter climatic conditions may produce decrease of plasma, perhaps as a protective measure. After thyroparathyroidectomy the red blood cell volume always fell steadily, presumably in part as a continuance of the initial drop with proper heating and diet, and in part as an effect of thyroid removal.

SUMMARY.

1. The plasma and serum calcium values of normal dogs are usually equal; occasionally higher values have been found for incinerated citrated plasmas, and these differences are being studied further.

2. If the cerebrospinal fluid can be correctly considered as that part of the plasma which can diffuse through an animal membrane, then the diffusible calcium of normal dog's *plasma* averages 53 per cent of the *serum* calcium. This figure is definitely lower than that found by dialysis methods with artificial semipermeable membranes for dog's *serum*.

3. The calcium in dog's plasma consists of a part in inorganic combination (averaging 53 per cent), and a part in specific organic combination (47 per cent); the latter only slowly dissociates on removal of calcium ions. After clotting it would appear that the organic compound is changed to another specific calcium organic compound, present in the serum, which dissociates to a greater extent, so that a larger percentage of the serum calcium is diffusible.

4. Removal of the parathyroids results in a decrease of the total blood calcium, and of both fractions. There is an apparent gradual increase in the proportion of *plasma* calcium that can diffuse, so that finally, in acute tetany, the figures for serum cal-

⁴ Cruickshank (45), p. 16.

cium and cerebrospinal fluid calcium tend to become exactly equal. This increase is, however, only apparent, since after parathyroidectomy the organic calcium compound with its calcium is more and more completely taken up by the clot, so that in acute tetany the serum calcium is less than the plasma calcium by from 3 to 4 mg. per 100 cc. As the blood calcium falls with the development of acute tetany the ratio between diffusible and non-diffusible calcium may remain approximately constant for at least 8 or 10 days.

5. It is suggested that the constancy of the blood calcium depends on the slightly dissociable organic calcium compound present in the plasma, which holds a definite amount of inorganic calcium in the plasma through a series of interlocked equilibria.

6. The direct cause of parathyroid tetany is due to the slight diminution (1 or 2 mg. per 100 cc.) in the inorganic calcium of the plasma, resulting indirectly from a diminution of the organic compound. The apparent inability to restore calcium to normal levels by feeding calcium compounds, is due, not only to the fact that the organic calcium is no longer estimated in the serum, but also to the fact that its diminished amount can no longer prevent some of the normal amount of inorganic calcium from escaping. This indicates, clinically, that in order to prevent tetany repeated small doses of calcium salts should be given at frequent intervals.

7. The nature of the organic compound is being studied.

8. A modification of Alport's method of determining calcium in whole blood, which utilizes the Kramer-Tisdall method, is described. It can be employed with 1 or 2 cc. of whole blood (prevented from clotting by addition of a small amount of saturated solution of ammonium carbonate), citrated or oxalated plasma, or cerebrospinal fluid, and gives nearly the same degree of accuracy as Tisdall's modification of the Kramer-Tisdall method.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

V. THE MOLECULAR WEIGHTS OF THE PROTEINS.*

PART 1. THE MINIMAL MOLECULAR WEIGHTS OF CERTAIN PROTEINS.

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* The results of this investigation were reported at the meeting of the American Society of Biological Chemists, at Washington, D. C., December, 1924 (11).

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I.

INTRODUCTION.

The large molecular weights of the proteins must be considered one of their outstanding characteristics, in great part responsible for such colloidal aspects of their behaviour as their failure to pass through many natural and artificial membranes. It is probably because of their size that proteins are retained by cell walls through which their constituent parts and their decomposition products freely pass. The cellular structure of biological systems, and their stability under changing metabolic conditions, depends, therefore, upon the separation and the concentration of large organic molecules within limiting membranes.

Many natural plant and animal cells were utilized in the development of our modern theory of solutions, because of the permeability of their cell walls to small organic and inorganic molecules, and their impermeability to such of their constituents as the proteins. In the classical paper before the Swedish Academy in which van't Hoff applied the gas laws to solutions by demonstrating the relation between osmotic pressure and the number of dissolved molecules, the argument rested in part upon De Vries' measurements of the plasmolysis of plant cells, in part upon Hamburger's measurements of the hemolysis of blood corpuscles, and in part upon Pfeffer's experiments with artificial

membranes (72). In the former, natural cells served as osmometers; in the latter, artificial cells, impermeable to certain molecules, allowed of the direct measurement of the osmotic pressures they produced.

Determination of Molecular Weights by Means of Osmotic Pressure Measurements.—Since natural and artificial cells could be used as delicate indicators of the osmotic pressure of solutions, it was natural to suppose that measurements of the osmotic pressures of such constituents of cells as the proteins might lead to exact knowledge of their molecular weights and volumes. Sørensen and his collaborators have recently determined the molecular weight of egg albumin (70), serum albumin, and serum globulin¹ with great accuracy by measurements of osmotic pressure. These investigators took into account sources of error involved in such measurements, which were not completely understood by earlier workers. These depend upon the reactivity and the multivalent character of the proteins, and upon the dissociation of the different compounds that proteins form with each other and with acids, bases, and salts.²

As a result of these complications, measurements of the osmotic pressures of proteins have not always led to consistent estimates of their molecular weights. The measurements on hemoglobin solutions of Reid, Hüfner and Gansser, Roaf, and of Adair illustrate this contention. Reid (66) "prepared the haemoglobin by crystallisation and obtained a pressure of about 4 millimetres of mercury for each 1 per cent. of haemoglobin"³ or a molecular weight of over 42,000. Hüfner and Gansser (41), on the other hand, found the molecular weight of horse hemoglobin to be approximately 15,000, and of ox hemoglobin 16,000. The great effect of the solvent upon the osmotic pressures of hemoglobin solutions was first indicated by Roaf (67), and has since been systematically studied for other proteins by Lillie (48), and for hemoglobin by Adair (3). In Roaf's investigation "three deter-

¹ Sørensen, S. P. L., personal communication.

² Different compounds of the same protein exert different pressures both because of the different number of particles into which they dissociate, and because of the complicated membrane equilibria which arise whenever the membrane is permeable to certain of the ions of such compounds, and impermeable to others (50).

³ Roaf (67), p. i.

minations of osmotic pressure were made, the first with distilled water, the second with 0.34 per cent. sodium bicarbonate (0.04 N) and the third with 0.2 per cent. sodium carbonate (0.04 N). The osmotic pressures obtained for 1 per cent. haemoglobin were 5.7, 5.3 and 11.6 millimetres of mercury respectively. These pressures correspond to 'aggregates' of 29787, 32035, and 14636."³

It should be noted that Roaf's lowest value for the molecular weight of hemoglobin in the presence of sodium carbonate was of the same order as Hüfner and Gansser's value. In this case "the haemoglobin 'aggregate' corresponds to its molecular weight calculated from the amount of iron present."³ Where the molecular weight is higher, it has been assumed that the hemoglobin existed in aggregates. Lillie's (48), Sørensen's (70), and Loeb's (50) investigations of the osmotic pressures of other proteins render it more probable that the lowest pressures correspond to those produced by the uncombined protein, while the higher pressures represent the dissociation of protein compounds with their attendant membrane equilibria.

Determination of Minimal Molecular Weights by Means of Combining and Containing Weights.—With the development of the modern theory of solutions, it was inevitable that the measurement of the osmotic pressures of protein solutions should seem the best method for the determination of their molecular weights. In the last 30 years such investigations have revealed complications which depended upon phenomena that are only now beginning to be understood, and that have thus far been adequately considered in but few researches. Meanwhile there has accumulated a vast body of analytical information regarding the composition of proteins on the basis of which their minimal molecular weights can be calculated, and a large number of physicochemical methods on the basis of which their equivalent combining weights can be determined.

In the succeeding sections of this paper the evidence that can be derived from the simultaneous consideration of the analytical and physical chemistry of the proteins will be considered. In a subsequent communication the relative size of the molecules of a series of proteins will be determined by the method of dialysis or ultrafiltration (7), and their probable molecular weights estimated as integral multiples of their

minimal molecular weights. This procedure of estimating molecular weights as multiples of minimal molecular weights has often been followed in classical inorganic and organic chemistry.

II.

Calculation of the Minimal Molecular Weights of Proteins from Their Composition.

Whereas great uncertainty exists concerning the true molecular weight of hemoglobin, the equivalent combining weight of this protein, and consequently its minimal molecular weight has been accurately known for 30 years. In 1894 Hüfner (39) studied the carbon monoxide-combining capacity of the hemoglobin of the ox, and found that 16,721 gm. of hemoglobin combined with each mol of carbon monoxide. Oxygen combines with the same amount of hemoglobin as does carbon monoxide (63). It is therefore certain that these important physiological reactions proceed stoichiometrically.

Iron Content of Hemoglobin.—Hemoglobin contains iron. Zinoffsky, Hüfner, Jaquet, and Abderhalden have determined the amount of iron contained in the hemoglobin of different species. Their results indicate that hemoglobin always contains between 0.335 and 0.40 per cent of iron, and at the same time suggest the first indirect method of estimating the minimal molecular weight of a protein in terms of a well known constituent of its molecule. For if the 0.335 per cent of iron in the hemoglobin molecule of the horse represents but 1 gm. atom of iron, the molecular weight of the protein must be approximately 16,669. The relation upon which calculations of this type depend is expressed by the equation

$$\text{Minimal molecular weight of protein} = \frac{\text{Atomic weight of element} \times 100}{\text{Per cent of element in protein}} \quad (1)$$

Precise estimates of minimal molecular weights, based upon actual determinations of the iron content of the hemoglobins of different species, are contained in Table I.

Copper Content of Hemocyanin.—The proteins of the blood of certain of the Mollusca and Arthropoda contain copper, which presumably subserves the same function as the iron of hemo-

globin. These proteins, because of their blue color, are all termed hemocyanins. The different amounts of copper in the hemocyanins of different genera and phyla indicate molecular weights that are not identical (64). Further investigation has demonstrated that these copper-containing proteins differ not only in their minimal molecular weights, but in many other respects (5).

Sulfur Content of Proteins.—Hemoglobin, hemocyanin, and many other proteins contain sulfur. Analyses of the sulfur contents of proteins, therefore, offer another method of estimating their minimal molecular weights. In the case of hemoglobin and hemocyanin, consideration of the sulfur content has for the most part confirmed the molecular weights postulated on the basis of the iron analysis. Occasionally, however, as in the case of the sulfur content of the hemoglobin of the ox, the sulfur analysis suggests that the molecular weight may be at least twice that demanded by the iron analysis.

Sulfide Sulfur Content of Proteins.—The large amount of sulfur that many proteins contain diminishes the usefulness of the analysis of total sulfur, for the smallest weights of proteins that can contain 1 atom of sulfur are often too small a fraction of their minimal molecular weights to aid in their estimation. Part of the sulfur in the protein molecule is, however, sulfide sulfur; estimated by means of its lead-blackening property (53). T. B. Osborne (58) has studied the ratio of sulfide sulfur to total sulfur in a series of proteins, and has shown that the sulfide sulfur usually represents an integral fraction of the total sulfur. On the basis of this relation, Osborne estimated that the molecular weights of many proteins were in the neighborhood of 30,000 (57).

The sulfide sulfur in the protein molecule has been supposed to represent, at least in part, the sulfur either in the amino acid cystine or cysteine. Cysteine contains but 1 atom of sulfur, while cystine contains 2. Whatever the nature of the sulfide sulfur, it represents an integral part of the protein molecule, and the quantities that analyses reveal can be used in estimating the minimal molecular weights of proteins, in the same manner as can their iron, copper, or total sulfur content.

Phosphorus Content of Proteins.—Certain proteins contain small amounts of phosphorus. Casein contains both sulfur and phosphorus, and the amounts of these two elements have led to

almost identical estimates of the minimal molecular weight of this protein at 4,220 and 4,372 respectively (47, 8).

Tryptophane Content of Proteins.—The tryptophane content of casein suggests that its molecular weight must be some multiple of the minimal molecular weight deduced from its phosphorus or sulfur content. Tryptophane exists in the casein molecule to the extent of 1.5 per cent according to the earlier analysts (59), 1.54 per cent according to Folin and Looney (21), and 1.7 per cent according to Dakin (16). The lower figure substituted in a relation similar to equation (1) leads to a molecular weight of 13,606; the higher, to 12,006. The average, 12,806, is three times the weight calculated from the phosphorus or from the sulfur content of casein.

A minimal molecular weight of this order enabled us, in a previous communication (14), to approximate the molecular composition of casein. This molecule contains but 2 molecules of histidine, 3 of arginine and phenylalanine, and 4 of aspartic acid and tyrosine. The amino acids present in larger amounts can also be calculated, but cannot be considered as giving further evidence regarding the size of the molecule. That the assumption of this number of amino acid molecules leads to a minimal molecular weight near 12,800 gm. is demonstrated by the calculation in Table IV.

Tryptophane is present in many protein molecules to a smaller extent than nearly any amino acid except cystine. Its quantitative isolation and estimation have, however, been difficult. In recent years numerous colorimetric methods have been evolved for determining the amount of this amino acid, as well as of tyrosine and of cystine, in protein hydrolysates. These methods avoid the losses that occur in the separation and quantitative estimation of amino acids. The excellent agreement between the tryptophane content of casein, as estimated by Folin and Looney, and the yields that have been obtained by isolation has already been alluded to.

Tyrosine and Cystine Contents of Proteins.—Folin and Looney (21) have estimated the tryptophane, tyrosine, and cystine contents of a large number of proteins, and a number of other investigators have estimated these amino acids in different proteins, either by these or by other colorimetric methods. Whether

cysteine or cystine exists in the protein molecule is still a debated question, but the form that is usually isolated from protein hydrolysates is cystine. If cysteine is present in the protein, the minimal molecular weight would, of course, be half that calculated from the cystine analyses.

Histidine, Arginine, and Lysine Contents of Proteins.—Van Slyke's (71) nitrogen distribution method of determining the diamino acids in proteins also avoids the losses often attendant upon the separation and gravimetric estimation of amino acids. Moreover, many proteins contain but a small number of molecules of either histidine, arginine, or lysine. Accordingly, we have recalculated his results from nitrogen as per cent of nitrogen to per cent of protein, and included them in our tabulation of significant data for the determination of molecular weights.⁴

The yields of certain amino acids, determined by the classical methods, have occasionally been included. The internal evidence offered by the data here presented is such that this procedure is less arbitrary than it may appear. For when the minimal molecular weights, calculated on the basis of a number of constituents of the same molecule, lead to values that are related to each other in definite and integral proportions, the results attain a significance which is greater than can be claimed for an individual analysis. Finally, if the equivalent combining weights of the proteins, determined by one of the physicochemical methods that we shall now consider, yield results that are also consistent with the minimal molecular weights, calculated by analytical methods, a body of evidence accumulates which can scarcely be considered fortuitous.

III.

Calculation of the Minimal Molecular Weights of Proteins from Their Equivalent Weights.

Saturation of Proteins with Gases.—Any stoichiometric reaction involving a protein may be utilized in estimating its equivalent weight. From the equivalent combining weight of the

⁴ D. Jordan Lloyd (49) has previously attempted to determine the molecular weight of gelatin from Van Slyke's data. Her method was different from that here employed.

reagent, the amount required to saturate or combine a given weight of the protein can be calculated by means of the relation

$$\frac{\text{Equivalent combining weight of protein}}{\text{Equivalent weight of reagent}} = \frac{\text{Weight of combined protein}}{\text{Weight of combined reagent}} \quad (2)$$

Certain proteins which are involved in the transport of oxygen in the body, such as the hemoglobins and the hemocyanins, combine chemically with oxygen, and some also with carbon monoxide. From the carbon monoxide-combining capacity of hemoglobin, Hüfner early estimated that its minimal molecular weight was 16,721 (39). His result was, as we have seen, in excellent agreement with the weight of hemoglobin that contained 1 atom of iron. As early as 1894 the minimal molecular weight of a protein had, therefore, been determined by the simultaneous consideration of analytical and physicochemical evidence.

Saturation of Proteins with Acids or Bases Determined by Electromotive Force Measurements.—Whereas only certain specialized proteins combine with gases, all proteins combine with acids or with bases. The amount of acid or base with which a protein can combine depends in the last analysis upon its composition and structure (12, 13, 25). Without reference to the nature of the groups involved in these reactions, the amount of combination at saturation of the protein can be utilized for the calculation of its equivalent combining weight, by means of the relation expressed in equation (2). The electromotive force methods for determining the maximum acid- or base-combining capacity of proteins have been considered in detail by Robertson (68), Pauli (62), and more recently by Hitchcock (34, 35), by Greenberg and Schmidt (27), and by us (12). Robertson determined in this way the equivalent combining weight for base of casein, serum globulin, and ovomucoid. A recalculation of Hitchcock's potential measurements has yielded the combining capacities of gelatin and edestin. Greenberg and Schmidt have also studied the base-combining capacity of gelatin, casein, and gliadin. These results have been considered in calculating minimal molecular weights wherever the protein did not contain so many groups as to lead to too low an equivalent weight.

Bracewell (9) also measured the acid-combining capacity of a few proteins in connection with his attempt to show that this

property depended upon their lysine and arginine, but not upon their histidine content. His method was less accurate than the electrometric procedure that has generally been employed. The reason for this will be considered in another place in connection with a study of the relation between the diamino acids in proteins and their acid-combining capacity.⁵ For completeness we have often included Bracewell's values, and considered them in the estimation of the equivalent combining weights of the proteins.

In the case of a few proteins the total number of acid or of basic groups is so low that their equivalent combining weights at saturation are of great value in the calculation of their minimal molecular weights. This is true of the acid groups in zein, gliadin, gelatin, and egg albumin, and of the basic groups in gliadin. In most proteins, however, the number of acid or basic groups is so large as to render measurements of their acid- or base-combining capacities at saturation almost valueless for this purpose.

Combination of Proteins with Acids and Bases Determined by Solubility Measurements.—Many proteins are very insoluble when uncombined with acid or base. Thus casein only dissolves in water to the extent of 0.11 gm., serum globulin to the extent of 0.07 gm. (11), and zein to the extent of 0.05 gm. in 1 liter (13). Certain of the globulins, the glutelins, and the prolamins, all of which are relatively insoluble in water, have very wide precipitation zones. These appear to form insoluble compounds with acids and with bases. This must occur in the case of proteins like zein and gliadin which do not pass into solution until very alkaline reactions are reached, but which nevertheless combine base at neutral reactions. It would appear that these proteins dissolve only after neutralization of all their base-combining groups. In one series of experiments with zein, the addition of two-thirds of the maximal base-combining capacity resulted in the solution of less than one-tenth of the protein present. The base must have combined with the zein to form one or more insoluble compounds.

T. B. Osborne (56) as early as 1902 studied the amount of acid required to form an insoluble hydrochloride of neutral edestin. He showed that the combining weight of protein, as

⁵ Cohn, E. J., and Berggren, R. E. L., unpublished data.

calculated from an analysis of the amount of acid in this compound, was a multiple of the minimal molecular weight calculated from its sulfur content. Here, again, the simultaneous consideration of the composition of a protein, and of its reactions, has yielded an accurate estimate of its minimal molecular weight.

When slightly larger amounts of acid or of base are added to certain proteins in the neighborhood of their isoelectric points, an amount of protein passes into solution which is proportional to the base added. Measurements of the solubility of edestin in systems containing protein in excess and known concentrations of base or of acid were also made by Osborne (56). More recently we have studied the solubility of casein and of serum globulin in such systems. The weight of protein dissolved in combination with the reagent can be calculated from the increased solubility produced by increments of base by means of equation (2). Only a few of the many free groups that such proteins contain usually dissociate in the neighborhood of their isoelectric points. Small amounts of reagent, therefore, combine with large amounts of the protein. As a result equivalent combining weights, calculated from solubility measurements, are of inestimable value in calculating the minimal molecular weights of slightly soluble proteins.

Up to the present, the equivalent weights only of casein and of serum globulin have been accurately determined by the solubility method. Bence-Jones' protein has also been studied to some extent, and the equivalent weights of other proteins should presently be available. Meanwhile it seemed desirable to report the methods that are being employed and the results that have thus far been obtained, together with the analytical information that has been collected.

IV.

Minimal Molecular Weights of Certain Proteins.

Hemoglobin.—Hemoglobin was the first protein whose minimal molecular weight was adequately determined. Moreover, five different methods have been employed in its estimation by a score of different investigators.

In the latter part of the last century, Zinoffsky and, later, Hüfner, and Jaquet (42) studied the iron and sulfur contents of

the hemoglobins of a large number of species. Their analytical results are summarized in Table I. In all of the six species considered, the hemoglobin contained between 0.335 and 0.40 per cent of iron. Taking the atomic weight of iron as 55.84, the weight of protein containing 1 atom of iron has been calculated by means of the relation

$$\text{Minimal molecular weight of Hb} = \frac{\text{Atomic weight of iron} \times 100}{\text{Per cent of iron in Hb}} \quad (3)$$

These analytical results indicate that the minimal molecular weights of the hemoglobins are not identical. The hemoglobin of the pig appears to have the smallest minimal molecular weight, 13,960, and of the horse and fowl the largest, 16,669. The minimal molecular weights of the hemoglobins of the ox and of the dog are essentially identical to the latter, or 16,619. This difference is, of course, smaller than the experimental error, and it might therefore be argued that all hemoglobins had the same molecular weight, and that the higher percentage of iron in the cat and pig were due to experimental error.

The sulfur contents of the hemoglobins of these same species not only give additional information regarding the minimal molecular weights of these proteins, but also demonstrate differences in the hemoglobins of different species quite as conclusively as the more recent investigations of Reichert and Brown (65) and of Landsteiner and Heidelberger (46). The weight of hemoglobin containing 1 atom of sulfur is in every case lower than the weight containing 1 atom of iron. As a result, the hemoglobin molecule must contain a larger number of atoms of sulfur than of iron. The ratio of iron atoms to sulfur atoms in the hemoglobin of the horse and of the pig is as 1:2. On the basis of 2 atoms of sulfur in horse hemoglobin, its molecular weight must be 16,878, which is in excellent agreement with the value calculated from its iron content, 16,669. The agreement in the case of pig hemoglobin is almost as satisfactory, although the minimal molecular weight would appear to be different. The ratio of iron atoms to sulfur atoms in the hemoglobin of the dog and of the cat is, however, as 1:3. The differences in these iron-sulfur ratios constitute conclusive evidence that the hemoglobins of these species cannot be identical.

The analyses of Hüfner and of Jaquet, which have elsewhere led to such consistent results, do not yield a simple ratio of iron atoms to sulfur atoms in the hemoglobin of the ox or of the fowl,

TABLE I.
Minimal Molecular Weights of the Hemoglobins.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		<i>per cent</i>	<i>gm.</i>		
Horse.	Iron content (73).	0.335	16,669	1	16,669
	Sulfide sulfur " (69).	0.190	16,878	1	16,878
	Sulfur " (73).	0.390	8,223	2	16,446
Pig.	Iron " (38).	0.40	13,960	1	13,960
	Sulfur " (38).	0.48	6,681	2	13,362
Cat.	Iron " (1).	0.35	15,954	1	15,954
	Sulfur " (1).	0.62	5,172	3	15,516
Ox.	CO-combining capacity (39).		16,721	2	33,442
	Iron content (39).	0.336	16,619	2	33,238
	Sulfur " (38).	0.45	7,127	5	35,635
	" " (54).	0.48	6,681	5	33,405
	Arginine " (71).	4.24	4,107	8	32,856
Fowl.	Iron " (42).	0.335	16,669	2	33,338
	Sulfur " (42).	0.86	3,729	9	33,561
Dog.	Iron " (42).	0.336	16,619	3	49,857
	Sulfide sulfur " (57).	0.335	9,573	5	47,865
	Sulfur " (42).	0.568	5,646	9	50,814

on the basis of a molecular weight in the neighborhood of 16,700.⁶ However, if it be assumed that the molecular weight is twice as great, or approximately 33,400, then the ratio of iron atoms to sulfur atoms in ox hemoglobin becomes 2:5 and in fowl hemo-

⁶ Müller (54) gives 0.48 per cent for the sulfur content of ox hemoglobin, referring presumably to an article of Hüfner's (39). In an earlier article (38) Hüfner gives the sulfur content of ox hemoglobin as 0.447.

globin 2:9. As a result, these two instances not only add further evidence regarding the specificity of the hemoglobins of different species, but indicate that the molecular weight of this protein must be greater than 16,700, at least in certain forms.

The carbon monoxide-combining capacity of ox hemoglobin also led Hüfner to a minimal molecular weight of 16,721 for this protein. This value is almost identical with that derived from its iron content, and Peters (63) has since demonstrated that the oxygen-combining capacity of hemoglobin was also proportional to its iron content.

The osmotic pressure determinations of Hüfner and Gansser (41) and of Roaf (67) that were carried out in alkaline solution indicated molecular weights of the order of 16,000. Roaf's determinations of the osmotic pressure of hemoglobin in distilled water and in sodium bicarbonate, however, indicated molecular weights of 29,787 and 32,035. Adair's recent measurements with horse and human hemoglobin, which have been corrected for the Donnan equilibrium, indicate still higher molecular weights. These results suggest, for reasons already indicated in the first sections of this paper, that the true molecular weight of hemoglobin is at least 33,400, and that the higher osmotic pressures that have occasionally led investigators to postulate lower molecular weights are to be explained as due either to dissociation of hemoglobin in alkaline solution, or to the membrane equilibria attending such dissociation.

There is evidence, however, that the molecular weight of certain hemoglobins may be higher than 33,400. Weymouth Reid (66) estimated the molecular weight of recrystallized dog hemoglobin by the osmotic pressure method. He obtained slightly higher osmotic pressures with twice recrystallized hemoglobin than with hemoglobin that had been recrystallized but once. Measurements upon the latter indicated a molecular weight of approximately 48,000, while his results with the twice recrystallized protein led to only a slightly lower value in the neighborhood of 42,000.

Osborne (57) has determined the sulfide sulfur in the hemoglobin of the dog. His estimate made in 1902 one "can probably rely upon . . . as reasonably accurate." The smallest weight

⁷ Personal communication.

of dog hemoglobin that can, on the basis of this determination, contain 1 gm. atom of sulfide sulfur would be 9,573 gm. The smallest molecular weight that can be postulated for dog hemoglobin on the basis of the simultaneous consideration of both the iron and the sulfide sulfur contents is five times the latter value, or approximately 47,865, a result which is in good agreement with that calculated from Reid's determination of the osmotic pressure of the same species. The analytical evidence becomes still more consistent if it be assumed that the dog hemoglobin molecule contains 4 atoms of iron, 7 of sulfide sulfur, and 12 of sulfur. The molecular weights calculated on this basis from these three hemoglobin constituents are 66,476, 67,011, and 67,752.⁸

As a result of studies of the oxygen-combining capacity of hemoglobin in the presence either of carbonic acid or of base, Adair has suggested that the molecule of hemoglobin may contain 4 atoms of iron and, therefore, have a molecular weight of 66,800.

Finally Van Slyke (71) has determined the arginine, histidine, and lysine contents of ox hemoglobin, and Hanke and Koessler (30) the histidine content of ox, horse, and cat hemoglobin. Only in the case of the arginine was the content of the amino acid low enough to yield a sufficiently high value for the weight of the protein containing 1 molecule, to be considered significant in the estimation of its molecular weight. The smallest weight of ox hemoglobin that can contain 1 molecule of arginine is 4,107 gm. Four times this value equals 16,428 gm., and eight times equals 32,856 gm., results which are in fair agreement with the molecular weight calculated above.

Hemocyanin.—Since the copper and sulfur contents of the hemocyanins of various forms are different, it follows that their molecular weights must also be different. The difference between the hemocyanins of certain Cephalopoda and Arthropoda is particularly striking. Thus the hemocyanin of *Octopus* contains more copper than does that of *Limulus*, while the latter

⁸ That the true molecular weight of the hemoglobins not only of the dog, but also of the ox and of the horse, is either 50,000 or 67,000 will be shown in a subsequent communication. Ultrafiltration and dialyzing experiments have shown that hemoglobin is larger than serum albumin (7), and that these three hemoglobins have very similar molecular dimensions.

contains nearly twice as much sulfur as the former. According to Henze (33) *Octopus* hemocyanin contains 0.38 per cent of copper. A calculation similar to that employed with the iron of hemoglobin leads to a molecular weight for this protein of 16,729. The sulfur content, 0.86 per cent, indicates that the smallest weight that can contain 1 atom of that element must be 3,729. Four times 3,729 leads to a much lower value for the minimal molecular weight than that estimated from its copper content. Five times 3,729 leads to a value that is too high. If these analytical values are assumed to be correct, the smallest

TABLE II.
Minimal Molecular Weights of the Hemocyanins.

	Method.	Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
<i>Octopus.</i>	Copper content (33).	0.38	16,729	2	33,458
	Sulfur " (33).	0.86	3,729	9	33,561
<i>Limulus.</i>	Copper " (5).	0.28	22,704	1	22,704
	Arginine " (71).	7.92	2,198	10	21,980
	Sulfur " (5).	1.56	2,056	11	22,616
	Lysine " (71).	7.16	2,040	11	22,440
	Histidine " (71).	7.82	1,983	11	21,813

molecular weight that can be attributed to this protein must be 33,500. For if it is assumed that this hemocyanin molecule contains 2 atoms of copper, its weight becomes 33,458; and if it contains 9 atoms of sulfur, it becomes 33,561. We may, therefore, take the minimal molecular weight of this protein as 33,500.

A similar calculation has been made on the basis of Alsberg and Clark's analysis (5) of copper and of sulfur in the hemocyanin of *Limulus*, and is reported in Table II. The histidine, arginine, and lysine contents of *Limulus* hemocyanin determined by Van Slyke (71) have also been used in this calculation. The minimal molecular weight of this substance is unquestionably different

from that of the hemocyanin of *Octopus*, as Alsberg maintained, and is probably 22,700.

For a summary of analyses and of containing weights of various hemocyanins, reference may be made to the article by Quagliariello (64).

Egg Albumin.—At the time these investigations were undertaken, egg albumin was the only protein whose molecular weight had been adequately estimated from the osmotic pressure which

TABLE III.
Minimal Molecular Weight of Egg Albumin.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
Osmotic pressure	(70).				34,000
Cystine	content (31).	0.865	27,769	1	27,769
"	" (43).	0.88	27,295	1	27,295
Tryptophane	" (21).	1.23	16,593	2	33,186
Histidine	" (60).	1.71	9,070	4	36,280
Sulfide sulfur	" (57).	0.491	6,532	5	32,660
Aspartic acid	" (60).	2.20	6,050	6	36,300
Tyrosine	" (21).	4.2	4,312	8	34,496
Lysine	" (60).	3.76	3,886	9	34,974
Arginine	" (60).	4.91	3,546	10	35,460
Phenylalanine	" (60).	5.07	3,256	10	32,560
Proline	" (60).	3.56	3,233	10	32,330
Sulfur	" (57).	1.616	1,984	17	33,728
Maximal base-combining capacity. ⁵			1,250	27	33,750

it produces in the neighborhood of its isoelectric point. As a result of a painstaking series of investigations, in which corrections for membrane equilibria were employed, S. P. L. Sørensen and his collaborators (70) came to the conclusion that the molecular weight of egg albumin was approximately 34,000.

Folin and Looney estimated the tryptophane and tyrosine contents of egg albumin as 1.23 and 4.2 per cent respectively. On the basis of the tryptophane content, the molecular weight of egg albumin must be at least 16,593. Twice this value, or 33,186,

is in excellent agreement with the molecular weight calculated by Sørensen from osmotic pressure measurements. A molecule of egg albumin that contains 8 molecules of tyrosine would, by a similar calculation, have a molecular weight of 34,496. Any difference in the assumed number of tyrosine molecules leads to a difference of over 4,000 in the calculated molecular weight. Therefore, this determination also confirms the estimate of the molecular weight of egg albumin.

Egg albumin also contains cystine, but Folin and Looney do not report a determination of the amount of this amino acid present. Harris (31) has estimated that egg albumin contains at least 0.865 per cent of cystine, and more recently Jones, Gersdorff, and Moeller (43) have found 0.88 per cent, using Folin and Looney's colorimetric method. On this basis, the molecular weight would be greater than 27,000, a result which cannot be considered as quantitatively satisfactory, but which nevertheless furnishes evidence of the size of this molecule.

There is more sulfide sulfur in egg albumin, as in edestin and casein, than can be accounted for on the basis of its cystine content. Osborne (57) has determined both the sulfide sulfur and the total sulfur contents. There is too large an amount of total sulfur in egg albumin to render its determination of great value in minimal molecular weight calculations. If, however, one assumes that the egg albumin molecule contains 5 atoms of sulfide sulfur, its molecular weight becomes 32,660. The fact that a molecule of this size contains 1 molecule of cystine or 2 of cysteine, and a prime number of sulfide sulfur and of sulfur atoms, makes the estimate of its minimal molecular weight independent of, but in excellent agreement with, Sørensen's osmotic pressure measurements.

In 1909 Osborne, Jones, and Leavenworth (60) completed an analysis of the hydrolytic products of this protein. Their results for histidine, arginine, aspartic acid, phenylalanine, and proline, which have been included in Table III, all support the conclusion of Sørensen, that the molecular weight of egg albumin is approximately 34,000. The tryptophane, tyrosine, and sulfur determinations render 33,800 the most probable value.

Casein.—The minimal molecular weight of casein also depends in large part upon its tryptophane content (21). Until recently the minimal molecular weight of casein has generally been assumed

to be approximately 4,300. This estimate has been based on its phosphorus and on its sulfur contents. In a recent investigation, Cohn and Hendry (14) have determined, by the solubility method, that the equivalent combining capacity of casein for NaOH, in the neighborhood of its isoelectric point, lies between 2,096 and 2,166 gm. In another investigation, Cohn and Berggren (12) have determined, by means of electromotive force measurements, that

TABLE IV.
Minimal Molecular Weight of Casein.

Method.			Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
			per cent	gm.		
Tryptophane	content	(21).	1.54	13,253	1	13,253
"	"	(16).	1.7	12,006	1	12,006
Histidine	"	(59).	2.50	6,204	2	12,408
"	"	(30).	2.84	5,461	2	10,922
Phosphorus	"	(8).	0.71	4,372	3	13,116
Sulfur	"	(29, 47).	0.76	4,220	3	12,660
Phenylalanine	"	(22).	3.88	4,255	3	12,765
Arginine	"	(59).	3.81	4,570	3	13,710
Tyrosine	"	(21).	5.36	3,379	4	13,516
"	"	(20).	5.77	3,139	4	12,556
Aspartic acid	"	(16).	4.1	3,246	4	12,984
Equivalent base-combining capacity		(14).		2,100	6	12,600
β -Hydroxyglutamic acid content		(16).	10.5	1,553	8	12,424
Ammonia	"	(59).	1.61	1,058	12	12,696
Cystine	"	(21).	0.25	96,080	1	96,080
"	"	(43).	0.26	92,384	1	92,384
Sulfide sulfur	"	(57).	0.101	31,752	3	95,256

the maximal base-combining capacity of casein was 546 gm. Four times the equivalent combining weight at saturation yields 2,184 gm., a value which is in good agreement with the equivalent combining weight for base near the isoelectric point.

The equivalent weight derived from solubility measurements is more accurate than that derived from electromotive force measurements. Six times the lower estimate derived from solubility

measurements equals 12,576, and six times the higher equals 12,996. The molecular weight derived from Folin and Looney's tryptophane determination is 13,253, and that derived from Dakin's gravimetric determination is 12,006. Six times the average of the limiting combining weights is 12,786, and of the tryptophane-containing weights is 12,629. Therefore, 12,800 may be taken as the minimal molecular weight of casein.

Besides tryptophane, casein contains several other amino acids in such small amounts that they render still more certain this estimate of its minimal molecular weight. A casein molecule of this size would contain 2 molecules of histidine, 3 of phenylalanine and arginine, and 4 of tyrosine and aspartic acid. The minimal molecular weight calculated from each of these component parts of the casein molecule falls between 12,400 and 13,700.

Two component parts of the casein molecule indicate that its true molecular weight must be larger than, and some multiple of, 12,800. Osborne (57) long since determined the sulfide sulfur content of casein. A molecule of casein containing but 1 atom of sulfide sulfur must weigh 31,752 gm. Folin and Looney (21) find as little as 0.25 per cent of cystine in casein, and Jones, Gersdorff, and Moeller (43), using the same method, have found almost precisely the same amount, 0.26 per cent. A molecule of casein containing 1 molecule of cystine, or 2 of cysteine, must therefore weigh approximately 96,000 gm. Such a molecule would contain 3 sulfide sulfur atoms, for three times 31,752 equals 95,256.

The sulfide sulfur and the cystine contents of casein indicate that its minimal molecular weight must be far greater than 12,800, the value at which we arrived from the consideration of the other amino acids that casein contains and from its combining capacity. If we multiply this accurately known minimal molecular weight by 7, the result is, however, not in very good agreement with the molecular weight postulated from the sulfide sulfur or the cystine content. Eight times the minimal molecular weight leads to a result which is in no better agreement. If we proceed tentatively on the basis of these analytical results, allowing ourselves to be persuaded of their accuracy because of their consistency, we must consider the molecular weight of casein as fifteen times 12,800, or 192,000. This estimate is precisely twice that demanded by

the cystine, or four times that demanded by the cysteine, content of this protein. We have long hesitated to assume so great a molecular weight as 192,000 for any protein.⁹ It is impossible, however, to reconcile these different analytical results on the basis of a smaller molecule.

Zein.—In considering the relation between the composition of zein and its acid and basic properties, we have recently had occasion to estimate its minimal molecular weight from a simultaneous consideration of its composition and its base-combining capacity.

"Cystine and histidine would seem to be the amino acids in which zein is poorest. If zein contains 0.8 per cent of histidine the molecular weight of zein cannot be less than 19,387. . . . There is some reason to believe that no very great change will occur in this estimate of the minimal molecular weight. For if the zein molecule contains 2 molecules of arginine its weight would be 19,344; 3 molecules of β -hydroxyglutamic acid bring the molecular weight to 19,752; 3 of aspartic acid to 22,182; and 4 atoms of sulfur to 21,380. Although it is probable that all of these values will change as methods of separation and analysis improve, the very high known percentage composition of zein on the one hand, and the frequency with which a small number of assumed molecules leads to a molecular weight near 20,000 allows us to assume a figure of this order for the minimal molecular weight of zein with a fair degree of probability" (13).

Measurements of the base-combining capacity of zein lead to an equivalent weight which is approximately one-sixth as great as the minimal molecular weight postulated on the basis of its histidine content. Two zein preparations were studied, one of which bound 0.00031 and the other 0.00028 mol of sodium hydroxide per gram (13). The equivalent weights of zein bound by 1 mol of sodium hydroxide were, therefore, 3,226 and 3,571 respectively. Six times the former leads to a minimal molecular weight of 19,356, and six times the latter combining weight to 21,426. These results must be considered in fair agreement with the minimal molecular weight estimated from analyses of zein.

"The true molecular weight of zein may be very much larger than 20,000. Folin and Looney have found only 0.5 per cent of cystine in zein hydrolysates by their colorimetric method. If this estimate is correct the molecular weight cannot be less than 48,040. Osborne had previously found that zein contained but a small amount of sulfide sulfur, and had accordingly predicted a high molecular weight. He found but 0.212 per cent of sulfide

⁹ See Cohn and Hendry (14), p. 549.

sulfur. On the basis of this determination 15,127 gm. of zein would contain but 1 such atom. Three times this value leads to a molecular weight of 45,381, which is in good agreement with the molecular weight postulated on the basis of the cystine content. This coincidence lends a certain weight to both values, and also suggests that all of the sulfide sulfur in zein cannot represent cystine" (13).

TABLE V.
Minimal Molecular Weight of Zein.

Method.			Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
			per cent	gm.		
Histidine	content	(61).	0.82	18,915	1	18,915
Arginine	"	(44).	1.82	9,566	2	19,132
Aspartic acid	"	(19).	1.8	7,394	3	22,182
β -Hydroxyglutamic acid	"	(19).	2.5	6,524	3	19,572
Sulfur	"	(57).	0.60	5,345	4	21,380
Tyrosine	"	(21).	5.6	3,234	6	19,404
Maximal base-combining capacity		(13).		3,400	6	20,400
Cystine	content	(43).	0.85	28,259	2	56,518
Histidine	"	(61).	0.82	18,915	3	56,745
Sulfide sulfur	"	(57).	0.212	15,127	4	60,508
Arginine	"	(44).	1.82	9,566	6	57,396
Aspartic acid	"	(19).	1.8	7,394	8	59,152
Sulfur	"	(57).	0.60	5,345	11	58,795
Cystine	"	(21).	0.5	48,040	2	96,080
Histidine	"	(61).	0.82	18,915	5	94,575
Sulfide sulfur	"	(57).	0.212	15,127	6	90,762
Arginine	"	(44).	1.82	9,566	10	95,660
Aspartic acid	"	(19).	1.8	7,394	13	96,122
Sulfur	"	(57).	0.60	5,345	18	96,210

Since these calculations were made, Jones, Gersdorff, and Moeller (43) have reported a cystine content in zein of 0.85 per cent. If this higher result is correct the cystine-containing weight is 28,259. Such a molecule would contain 2, rather than 3, sulfide sulfur atoms.

In zein, as in casein, the cystine content suggests a higher molecular weight than does any other amino acid. For neither

of the minimal molecular weights calculated from these different analyses of cystine is an integral multiple of 19,400. Jones, Gersdorff, and Moeller's cystine determination leads to a minimal molecular weight of approximately 58,000; Folin and Looney's to one of 96,000. Both would contain 2 cystine or 4 cysteine molecules, while the former would contain 3, the latter 5, histidine molecules. The physicochemical and analytical data have been calculated and tabulated on both bases. The weights of zein containing sulfur and aspartic acid, which in the first computation led to slightly abnormal values, conform admirably to a minimal molecular weight of approximately 58,000 or 96,000. Nevertheless the discrepancy between the cystine estimates is such that it seems preferable, pending its redetermination, to consider 19,400 as the minimal molecular weight of zein.

Gliadin.—The minimal molecular weight of gliadin can be estimated with great precision both by analytical and by physicochemical means. The very small number of free acid and free basic groups in the prolamins renders electromotive force measurements of their equivalent weights particularly valuable in the estimation of their minimal molecular weights.

The tryptophane content of gliadin has been determined both gravimetrically and colorimetrically. Abderhalden and Samuely (2) found approximately 1 per cent of tryptophane in gliadin, Folin and Looney (21) estimated that a slightly larger amount was present, namely 1.14 per cent, and Cross and Swain (15) found 1.11, 1.19, 1.03, and 1.13 per cent in preparations from different flours. May and Rose (52), using a comparative colorimetric method based upon the amount of tryptophane in casein, estimated that gliadin contained 1.05 per cent. Since casein probably contains nearer 1.6 than 1.5 per cent, their results are probably low by about 6 per cent. These four different investigations leave little doubt that gliadin contains between 1.0 and 1.14 per cent of tryptophane. The minimal molecular weight of gliadin calculated from the lowest value is 20,410, and from 1.14 per cent is 17,904.¹⁰

¹⁰ If Folin and Looney's tryptophane and cystine estimates are both correct, the molecular weight of gliadin cannot be less than 72,000. The sulfur content of gliadin leads to a containing weight which also suggests 72,000 rather than 20,700, but the lysine content would demand a molecule of twice this size.

The lysine content of gliadin is also very small. Van Slyke's most recently published determination of this quantity, 0.69 per cent (71),¹¹ leads to a minimal molecular weight of 21,173. The tryptophane and lysine contents of gliadin, therefore, suggest a minimal molecular weight in the neighborhood of 20,000.

A number of other amino acids are present in gliadin in very

TABLE VI.
Minimal Molecular Weight of Gliadin.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
Tryptophane	content (21).	1.14	17,904	1	17,904
"	" (2).	1.0	20,410	1	20,410
Lysine	" (71).	0.69	21,173	1	21,173
Cystine	" (21).	2.32	10,353	2	20,706
β -Hydroxyglutamic acid	" (17).	2.4	6,796	3	20,388
Sulfide sulfur	" (57).	0.619	5,181	4	20,724
Histidine	" (71).	3.35	4,630	4	18,520
Arginine	" (71).	3.14	5,544	4	22,176
Tyrosine	" (21).	3.5	5,174	4	20,696
Maximal base-combining capacity	(25).		3,333	6	20,000
"	"		3,448	6	20,688
Sulfur content	(57).	1.027	3,123	7	21,861

small amount. Thus, Folin and Looney's cystine determination (21) leads to a minimal molecular weight of 10,353, and Jones, Gersdorff, and Moeller's (43) lower determinations, to slightly higher values. On the basis of Folin and Looney's estimate, 2 molecules of cystine, or 4 of cysteine lead to a minimal molecular weight of 20,706; 3 of β -hydroxyglutamic acid, to 20,388; and

¹¹ Cross and Swain have used Van Slyke's method in estimating the diamino acids. Whereas the sum of the average results for all these acids is equal to the sum of the histidine, arginine, and lysine determinations of Van Slyke, the variations between their different analyses were so great as to preclude their use in the calculation of containing weights. Their "tyrosine values are doubtless a little high owing to the residual color of the reagent" (15), and are therefore not considered.

4 of tyrosine, to 20,696. Clearly the minimal molecular weight of this protein lies between 20,000 and 21,000, and most probably in the neighborhood of 20,700.

The relation between the sulfide sulfur and the cystine in gliadin is precisely what should be expected provided all of the sulfide sulfur were present as cystine. A molecule of gliadin containing 2 molecules of cystine should weigh 20,706. A molecule containing 4 atoms of sulfide sulfur should weigh 20,724. The agreement is excellent, and is the more noteworthy since we have seen that many proteins contain more sulfide sulfur than can be accounted for on this basis.

Both the acid- and the base-combining capacities of gliadin have been measured. Bracewell (9) estimated that 1 gm. of gliadin combined with 0.00028 mol of hydrochloric acid. Electromotive force measurements made by one of us in 1917 and measurements made more recently in this laboratory suggest that Bracewell did not obtain the maximal acid-combining capacity of this protein.

Greenberg and Schmidt (25) have recently measured the base-combining capacity of gliadin. They find that 1 gm. of gliadin combines with 0.00030 mol of base. The equivalent weight to which this estimate of the base-combining capacity leads is 3,333 and the minimal molecular weight, 20,000. The method of calculation employed by Greenberg and Schmidt (27) is likely to have led to too low a value for the equivalent weight. Miss Berggren, working in this laboratory, and using the method of calculation that has elsewhere been explained (12), has studied two gliadin preparations, and come respectively, to 0.00028 and 0.00030 as the base-combining capacity per gram. The average leads to a still more concordant estimate of the minimal molecular weight of gliadin.

Glutenin.—Up to the present, information concerning the minimal molecular weight of glutenin depends entirely upon analytical evidence and largely upon the colorimetric determinations of tryptophane, tyrosine, and cystine. These, however, are very consistent with each other and with Dakin's estimate (17) of the amount of β -hydroxyglutamic acid in glutenin. It is to be hoped, however, that solubility and electromotive force studies upon this protein will be carried out in the near future.

Folin and Looney (21) have found 1.8 per cent, and Jones, Gersdorff, and Moeller (43) 1.56 per cent of cystine in glutenin. The corresponding containing weights are 13,344 and 15,397. The divergence between these results is sufficient to render the cystine content of glutenin less useful in the estimation of its minimal molecular weight than its tryptophane or tyrosine content. Folin and Looney have found 1.68 per cent of tryptophane in glutenin, and Cross and Swain (15) have found slightly smaller amounts, namely 1.59, 1.61, 1.55, and 1.61 per cent, in glutenins derived from four different flours. The minimal molecular weight of glutenin calculated from the highest estimate of its tryptophane content is 12,149. That calculated from its tyrosine content is

TABLE VII.
Minimal Molecular Weight of Glutenin.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
Tryptophane	content (21).	1.68	12,149	3	36,447
β -Hydroxyglutamic acid	" (17).	1.8	9,061	4	36,244
Tyrosine	" (21).	4.5	4,024	9	36,216
Cystine	" (21).	1.80	13,344		
"	" (43).	1.56	15,397		

4,024. A molecule containing 3 molecules of tyrosine would therefore weigh 12,072. Therefore, 12,100 may be taken as the minimal molecular weight on the basis of these amino acids.

Dakin (17) has determined the amount of β -hydroxyglutamic acid in glutenin. On the basis of his estimate, a molecule of glutenin containing 1 molecule of this acid would weigh 9,061 gm. The smallest molecule that can be attributed to glutenin on the basis of Folin and Looney's and Dakin's measurements would therefore contain 3 molecules of tryptophane, 4 of β -hydroxyglutamic acid, and 9 of tyrosine. On the basis of 4 β -hydroxyglutamic acid molecules, the gliadin molecule would weigh 36,244, or almost precisely three times the previously pos-

tulated minimal molecular weight of 12,100. Therefore, 36,300 may tentatively be taken as the minimal molecular weight of this protein.

Gelatin.—Upon a consideration of the diamino acids in gelatin, Lloyd (49) has estimated that its molecular weight is approximately 10,300. Both analytical and physicochemical evidence favors a minimal molecular weight of this order.

Gelatin contains neither tryptophane, tyrosine, nor β -hydroxyglutamic acid, three of the amino acids whose determination has

TABLE VIII.
Minimal Molecular Weight of Gelatin.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
Phenylalanine content	(18).	1.4	11,793	1	11,793
Histidine	" (71).	2.94	5,276	2	10,552
Aspartic acid	" (18).	3.4	3,915	3	11,745
Ammonia	" (18).	0.4	4,258	3	12,774
"	" (71).	0.49	3,476	3	10,428
Lysine	" (71).	5.92	2,468	4	9,872
Glutamic acid	" (18).	5.8	2,536	4	10,144
Arginine	" (71).	8.22	2,118	5	10,590
Leucine	" (18).	7.1	1,846	6	11,076
Maximal base-combining capacity	(25).		1,666	6	10,000
" " "	(36).		1,786	6	10,716
" acid-combining "	(35).		1,124	9	10,116

led to the most satisfactory estimates of the molecular weights of other proteins.

Cystine is present in the gelatin molecule to a smaller extent than is any other amino acid. Folín and Looney (21) found only 0.2 per cent of cystine by their colorimetric method, and Jones, Gersdorff, and Moeller (43) found slightly smaller amounts, 0.15 and 0.16 per cent in two preparations, but a larger amount, 0.31 per cent, in Holland gelatin. The weights of gelatin that would contain 0.31, 0.20, and 0.15 per cent of cystine are 77,484, 120,100, and 160,133. Provided cysteine rather than cystine obtained in the native

protein, the minimal molecular weights calculated from these analyses would be 38,742, 60,050, and 80,067. The fact that these figures differ from each other by approximately 20,000 is perhaps not without significance. The variation between the different preparations is such, however, that the calculation must for the present rest on the other components of the gelatin molecule.

Several amino acids are present in gelatin in small amounts. The phenylalanine content leads to a molecular weight of 11,793. The weight of gelatin containing 2 histidine molecules, 3 molecules of aspartic acid and of ammonia, 4 of glutamic acid and of lysine, 5 of arginine, and 6 of leucine, all lead to minimal molecular weights between 9,872 and 11,800. The variation between the minimal molecular weights estimated on the basis of these different determinations is, however, rather greater than in many of the proteins we have considered. Whether this is due to the absence of those amino acids that can most accurately be determined, or to inherent variability in the material cannot be decided at this time.

Electromotive force measurements of Hitchcock (36) and of Greenberg and Schmidt (25) upon systems containing gelatin and sodium hydroxide, and gelatin and hydrochloric acid, yield more satisfactory evidence for the minimal molecular weight of this protein. A recalculation of the potential measurements of Hitchcock's titration curve indicates that he reached reactions at which the gelatin was saturated with base. Apparently each gram of gelatin in his experiments combined 0.00056 mol of base. Greenberg and Schmidt report the base-combining capacity of gelatin as 0.00060. The equivalent weights corresponding to these values are 1,786 and 1,666 respectively. If it be assumed that the alkali was bound by six acid groups, the minimal molecular weight becomes 10,716 and 10,000 respectively. Hitchcock (35) has also measured the acid-combining capacity of gelatin. The equivalent weight of gelatin for acid calculated from this quantity is 1,124, a value precisely two-thirds as great as the combining weight for base. If gelatin, therefore, is considered to contain nine basic groups, the minimal molecular weight becomes 10,116.

These three physicochemical investigations,¹² therefore, lead to results that are consistent with each other, with analytical evidence, and with a minimal molecular weight in the neighborhood of 10,300. The very low percentage of cystine in gelatin suggests that the molecular weight must be at least four, and maybe sixteen, times the minimal molecular weight.

Edestin.—The early investigations of Osborne led him to believe that "the molecular weight of edestin must be about 14,258, or a multiple of this."¹³ The measurements upon which Osborne based this estimate were of two kinds. The first depended upon observations made in the course of the preparation of crystalline edestin. Osborne noted that not only was neutral edestin relatively insoluble, but that it formed an insoluble hydrochloride. Analyses revealed the fact that edestin combined with as much alkali to form a soluble basic salt as acid to form the insoluble hydrochloride. The composition of the latter compound yielded 14,258 as the equivalent combining weight of edestin and hydrochloric acid.

The sulfur content of edestin confirmed Osborne in his belief that its molecular weight "must be about 14,258, or some multiple of this." The smallest weight of edestin that can contain 1 atom of sulfur is 3,644 gm. Four times 3,644 equals 14,576, a value in excellent agreement with the minimal molecular weight of edestin calculated from the composition of its insoluble acid compound.

Folin and Looney (21) have determined the tryptophane, tyrosine, and cystine contents of edestin. The minimal molecular weight calculated from the tryptophane content is 14,578, or almost exactly Osborne's early estimate. These analytical results, one upon a compound of edestin, one upon an elementary constituent, and one upon an amino acid constituent, thus yield 14,258, 14,576, and 14,578 as combining and containing weights respectively, and leave little doubt that 14,500 is the approximate minimal molecular weight.

¹² The measurements of Smith (Smith, C. R., *J. Am. Chem. Soc.*, 1921, xliii, 1350) upon the osmotic pressure of gelatin solutions, had been overlooked. Smith's estimate of 96,000 must be considered as the lower limit for the molecular weight of this substance. The probable molecular weight thus becomes 123,600 or 164,800 (note added to proof).

¹³ Osborne (56), p. 55.

Cystine is present in edestin in a very small amount. Folin and Looney found 0.75 per cent and Jones, Gersdorff, and Moeller, 0.97 per cent. The agreement is not good, but the results indicate that the cystine-containing weight would be approximately double the minimal molecular weight. The cysteine-containing weight would therefore be approximately equal to the tryptophane-containing weight.

That the minimal molecular weight of edestin was at least twice the value calculated from the composition of insoluble edestin hydrochloride and from its tryptophane content, was

TABLE IX.
Minimal Molecular Weight of Edestin.

Method.			Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
			per cent	gm.		
Cystine	content	(21).	0.75	32,027	1	
"	"	(43).	0.97	24,763	1	
Tryptophane	"	(21).	1.40	14,578	2	29,156
Equivalent acid-combining capacity			(56).	14,258	2	28,516
Sulfide sulfur content			(57).	9,269	3	27,807
Histidine	"	(30).	3.04	5,102	6	30,612
"	"	(71).	3.92	3,954	7	27,678
Sulfur	"	(58).	0.88	3,644	8	29,152
Lysine	"	(71).	3.76	3,886	8	31,088
Tyrosine	"	(21).	5.70	3,177	9	28,593

indicated by the sulfide sulfur determination of Osborne (57). The minimal molecular weight calculated from the sulfide sulfur content is 9,269. Three times this weight is 27,807, or approximately double the minimal molecular weight.

The evidence that has been examined thus far leaves little doubt that the minimal molecular weight of edestin is approximately 29,000.

Tyrosine, though present in relatively large amount, and, therefore, yielding a comparatively low containing weight, also leads to a molecular weight near 29,000. The histidine determinations

of Van Slyke (71), and of Hanke and Koessler (30), have also been included in Table IX, although the results indicate either slight analytical errors or a molecular weight of twice this magnitude.

The combining capacity of edestin has been estimated both by the solubility method and by electromotive force measurements. Osborne (56) studied the solubility of edestin both in systems containing sodium hydroxide and in systems containing hydrochloric acid. Either the complications that always obtain when this method is applied to globulins,—and upon which their classification in all probability depends,—interfered with the accuracy of these measurements, or equilibrium was not obtained in these systems. The amount of edestin carried into solution by increasing amounts both of acid and of base were not very constant. The average results indicate, however, that as much acid was required to dissolve edestin and edestin hydrochloride, as had been required to form the insoluble compound.

The electromotive force measurements that have been made upon edestin (34), although themselves very accurate, are of no value for the determination of the molecular weight, since this protein is particularly rich in both acid and basic groups. The relation between the number of such groups and the amphoteric properties of this protein will therefore be considered in another place.

Serum Globulin.—Among the globulins, solubility studies are usually complicated by the presence of more than one saturating body. Nevertheless, the very large solvent action of small amounts of alkali renders the equivalent combining weights of these proteins, as determined by the solubility method, of particular importance.

Edestin, as Osborne (56) demonstrated, is not only relatively insoluble itself, but also forms a relatively insoluble acid compound. Our experiments suggest that serum globulin may behave in a comparable manner. The behaviour of the serum globulins may prove even more intricate, if Sørensen's hypothesis prove correct, that every preparation, no matter how carefully fractionated, is a combination of euglobulin and pseudoglobulin.

In our experience, after small amounts of base have been added

to serum globulin,¹⁴ subsequent increments of base carried equivalent amounts of protein into solution. Three experiments that are in fair agreement with each other and that were carried out with different preparations, are reported in Table X. The difference in solubility of globulins in two systems containing different amounts of base, but identical in every other respect, is given in next to the last column. From this the globulin carried into solution per mol of base has been calculated (in the last column). Solubility was estimated by the nitrogen content

TABLE X.

Equivalent Combining Weight of Serum Globulin and NaOH, Estimated from Solubility Measurements.

Preparation No.	Experiment No.	Globulin in system.	NaOH added to globulin in 50 cc. Mols $\times 10^{-3}$				Globulin dissolved by	
			0.25	0.50	1.00	2.00	Added NaOH.	1 mol NaOH.
		gm.	Globulin dissolved: gm.				gm.	gm.
IVa ₂	8	0.1371	0.0382	0.0726	0.1417	0.2776	0.0344	13,760
	8	0.5482					0.1359	13,590
VIa	117	0.2366		0.0786	0.1584		0.0798	15,960
	117	0.5884		0.0394	0.1107		0.0713	14,260
	117	0.5884			0.1107	0.2401	0.1294	12,940
	117	0.5884		0.0394		0.2401	0.2007	13,380
VIIa	120	0.350		0.0596	0.1372		0.0776	15,520
	120	0.560		0.0615	0.1341		0.0726	14,520
Average.....								14,241

of aliquot parts of the filtrate, but is reported as grams of protein dissolved in the systems. The small volume occupied by the precipitate has been neglected, although this may have intro-

¹⁴ These globulin fractions were prepared in the manner that has elsewhere been described in detail (10). It is noteworthy that, whereas the amount of the saturating body had no effect upon the amount of Preparation IVa₂ or of VIIa dissolved by sodium hydroxide, the amount of VIa dissolved by the first additions of sodium hydroxide was smaller the larger the amount of saturating body. This phenomenon is probably to be attributed to the formation or dissociation, in experiments with VIa, of an insoluble compound, before solubility appreciably increased.

duced a small error. In these experiments between 12,900 and 15,960 gm. of serum globulin were dissolved by each mol of sodium hydroxide. Probably 14,000 best represents the equivalent combining weight for base of this protein in the neighborhood of its isoelectric point.

Serum globulin contains tryptophane. Folin and Looney (21) have estimated that serum globulin contains 2.28 per cent of this amino acid. The smallest weight of this protein that can

TABLE XI.
Minimal Molecular Weight of Serum Globulin.

Method.	Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
	per cent	gm.		
Equivalent base-combining capacity.		14,000	2	28,000
Tryptophane content (21).	2.28	8,952	3	26,856
Sulfide sulfur " (69).	0.63	5,090	5	25,450
Sulfur " (29).	1.11	2,889	9	26,001
" " (69).	1.38	2,324	12	27,888
Tyrosine " (21).	6.7	2,703	10	27,030
Maximal base-combining capacity (68).		2,128	13	27,664

contain 1 molecule of tryptophane is, therefore, 8,952 gm. Three times 8,952 equals 26,856. The solubility measurements indicated that 1 mol of sodium hydroxide dissolved approximately 14,000 gm. of serum globulin. Twice this weight is 28,000. Therefore, 27,000 or 27,500 may be taken as the minimal molecular weight of this protein.

After these calculations had been made, S. P. L. Sørensen¹⁵

¹⁵ At a meeting of the National Academy of Science at Cambridge, Mass., Nov. 11, 1924, we reported the minimal molecular weight of serum globulin as 27,500, and the most probable molecular weight suggested by dialysis and ultrafiltration experiments as three times this, or 82,500. At the same time we gave the minimal molecular weight of serum albumin as 5,000. Since dialysis experiments showed the albumin of serum to be larger than that of egg, we suggested as its probable molecular weight, 40,000, or a slightly greater multiple of 5,000.

After the meeting, Professor Sørensen, who was present, informed us of

kindly brought to our attention recent unpublished measurements of the osmotic pressure of serum globulin and of serum albumin. As the result of experiments of the same kind that Sørensen and his collaborators had made upon egg albumin (70), they have come to the conclusion that "the molecular weight of the serum globulins will vary from about 80,000 to about 140,000." Three times 27,000, the minimal molecular weight that we arrived at on the basis of solubility measurements, and of the tryptophane, tyrosine, histidine, and arginine contents of serum globulin, yields 81,000, and five times the minimal molecular weight yields 135,000, values which are in excellent agreement with the molecular weight estimated by Sørensen from osmotic pressure measurements. Sørensen's theory suggests that the lower may be considered as the molecular weight of pseudoglobulin and the higher of euglobulin.

Serum Albumin.—Considering the physiological significance of this protein and the excellent methods for its crystallization, there has been amassed very little quantitative information regarding its composition. Probably its sulfur and sulfide sulfur contents are well known. The weight of this protein that should contain 1 atom of sulfur is 1,697 gm. and of sulfide sulfur is 2,505 gm., according to Schulz's determinations (69). Of these two quantities, three times the smaller weight is 5,091, and twice the larger weight is 5,010. These two estimates agree to 0.2 per cent and suggest that the molecular weight must be a multiple of 5,000.

At the time that these investigations were nearing completion, Sørensen brought his new osmotic pressure determination of the molecular weight of this protein to our attention.¹⁵ His estimate of 45,000 is precisely nine times the minimal molecular weight calculated from the sulfur and sulfide sulfur contents of serum albumin.

Hartley (32) has determined the arginine, histidine, and lysine contents of serum albumin by Van Slyke's nitrogen distribution method, and his results are consistent with a molecular weight

new, unpublished work from his laboratory, and gave us his manuscript, "On the osmotic pressure of proteins," with permission to refer to his results in this communication. That methods as different as those that he has employed and that are reported here have led to such similar results is a source of satisfaction.

of 45,000. Harris (31) has recently published an estimate of the cystine in this protein, so large that there is not enough sulfide sulfur in the molecule to account for it. Jones, Gersdorff, and Moeller (43), using Folin and Looney's colorimetric method, found a much smaller amount; namely, 2.88 per cent. The corresponding containing weight, 8,340, can only be reconciled with a molecular weight of 45,000, if it be assumed that cysteine, rather than cystine, exists in the protein molecule.

Although information regarding the amino acid composition of serum albumin is so unsatisfactory, the excellent agreement between the minimal molecular weight deduced from Schulz's old determination of sulfur and sulfide sulfur, and Sørensen's new

TABLE XII.
Minimal Molecular Weight of Serum Albumin.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
Osmotic pressure. ¹					45,000
Cystine	content (43).	2.88	8,340	5	41,700
Histidine	" (32).	3.40	4,562	10	45,620
Cystine	" (31).	6.45	3,724	12	44,688
Arginine	" (32).	4.90	3,553	13	46,189
Sulfide sulfur	" (69).	1.28	2,505	18	45,090
Sulfur	" (69).	1.89	1,697	27	45,819
Lysine	" (32).	13.20	1,107	41	45,387

osmotic pressure measurements leave little doubt that 45,000 is the true molecular weight of this protein.

Fibrin.—Fibrin is the last of the normal protein constituents of mammalian blood to be considered. Its sulfur and sulfide sulfur have been estimated, the former by Hammarsten (28). Folin and Looney (21) have made colorimetric determinations of tryptophane, tyrosine, and cystine, and Van Slyke (71) has determined histidine, arginine, and lysine by his nitrogen distribution method. Most of the constituents of proteins that are usually present in very small amounts have thus been analyzed, but not

one of these leads, by itself, to a minimal molecular weight greater than 9,000. The results of these different measurements are not always consistent, however, with a low minimal molecular weight, and suggest either that the molecular weight is relatively high, or that the preparations studied were not identical.

The first inconsistency appears when we consider the sulfide

TABLE XIII.
Minimal Molecular Weight of Fibrin.

Method.		Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
		per cent	gm.		
Sulfide sulfur content	(45).	0.38	8,439	2	16,878
Cystine	" (21).	3.5	6,863	2	13,726
Tryptophane	" (21).	2.9	7,038	2	14,076
Histidine	" (71).	2.98	5,205	3	15,615
Sulfur	" (28).	1.10	2,915	5	14,575
"	" (45).	1.2	2,672	5	13,360
Tyrosine	" (21).	6.5	2,786	5	13,930
Arginine	" (71).	7.29	2,388	6	14,328
Lysine	" (71).	10.14	1,441	10	14,410
Maximal acid-combining capacity (9).			1,042	14	14,588
Sulfide sulfur content	(45).	0.38	8,439	5	42,195
Cystine	" (21).	3.5	6,863	6	41,178
Tryptophane	" (21).	2.9	7,038	6	42,228
Histidine	" (71).	2.98	5,205	8	41,640
Sulfur	" (28).	1.10	2,915	15	43,725
"	" (45).	1.2	2,672	16	42,752
Tyrosine	" (21).	6.5	2,786	15	41,790
Arginine	" (71).	7.29	2,388	18	42,984
Lysine	" (71).	10.14	1,441	29	41,789
Maximal acid-combining capacity (9).			1,042	42	43,764

sulfur and cystine contents that have been reported for this protein. Either the sulfide sulfur of Krüger (45) is abnormally low, or Folin and Looney's estimate of cystine, 3.5 per cent, is high, for not enough sulfide sulfur has been estimated to account for the cystine. Jones, Gersdorff, and Moeller (43) have reported a slightly higher cystine content, 3.72 per cent.

The three determinations of Folin and Looney are in good agreement with each other. Thus if fibrin is assumed to contain 2 cystine molecules, its molecular weight becomes 13,726, while 2 tryptophane molecules bring the weight to 14,076, and 5 tyrosine molecules to 13,930. These results suggest a minimal molecular weight of 14,000 for the preparation studied.

The arginine and the lysine contents, determined by Van Slyke (71), are also in good agreement with each other and with Hammarsten's old sulfur content, but lead to a minimal molecular weight in the neighborhood of 14,400, slightly higher than that derived from Folin and Looney's determination. Not only does this discrepancy appear on the basis of a minimal molecular weight of this order, but the sulfide sulfur and histidine also lead to very abnormal results.

These irregularities disappear in large part if it be assumed that the minimal molecular weight of fibrin is three times 14,000, or 42,000. In Table XIII the analytical data have been calculated on both the lower and higher bases. The more consistent results when 42,000 is assumed as the minimal molecular weight would seem to depend upon more than mere statistical probability. The relatively good agreement between this minimal molecular weight as calculated from cystine and from sulfide sulfur does not, of course, explain how 6 molecules of cystine can contain 5 atoms of sulfide sulfur. Although it is very desirable that a new analysis of fibrin be undertaken upon a carefully purified preparation, it seems reasonably certain from Folin and Looney's measurements that the minimal molecular weight cannot be smaller than 14,000, and that it is probably three times 14,000, or 42,000.

Bence-Jones' Protein.—The protein that appears in the urine in cases of Bence-Jones' proteinuria has now been known for three-quarters of a century, and has been studied by a number of different investigators. It can be purified by crystallization in the neighborhood of its isoelectric point, although the preparations that have been studied have generally been precipitated in an amorphous condition. Whether the different analytical (37, 51) and anaphylactic results (6) that have been reported from the study of material derived from different sources, depend upon impurity of the preparations, or upon the existence of more than one protein exhibiting the characteristic reactions of Bence-

Jones' protein toward electrolytes at high temperature, cannot be answered at this time. The following calculations reveal both analytical inconsistencies and analytical criteria.

The appearance of Bence-Jones' protein in the urine invests the estimation of its molecular weight with special interest. Provided it is excreted unchanged by the normal kidney, its molecular weight may be expected to give information concerning the permeability of that organ.

The recent estimates of the tryptophane and tyrosine contents

TABLE XIV.
Minimal Molecular Weight of Bence-Jones' Protein.

Method.	Amount of constituent present.	Weight combining or containing 1 atom or molecule.	Assumed No. of atoms or molecules.	Minimal molecular weight.
	<i>per cent</i>	<i>gm.</i>		
Tryptophane content (21).	1.67	12,222	1	12,222
Aspartic acid " (37).	2.17	6,134	2	12,268
Proline " (37).	2.71	4,247	3	12,741
Phenylalanine " (37).	4.92	3,356	4	13,424
Arginine " (37).	6.06	2,872	4	11,488
Tyrosine " (21).	7.36	2,461	5	12,305
Amide nitrogen " (37).	1.30	1,077	12	12,924
Tryptophane " (21).	1.67	12,222	2	24,444
Aspartic acid " (37).	2.17	6,134	4	24,532
Phenylalanine " (37).	4.92	3,356	7	23,492
Sulfur " (37).	1.18	2,718	9	24,462
Tyrosine " (21).	7.36	2,461	10	24,610

of Bence-Jones' protein suggest that its minimal molecular weight is approximately 12,250. Folin and Looney's (21) measurements yield the results that a molecule of this protein containing 1 molecule of tryptophane should weigh 12,222 gm., and 1 containing 5 molecules of tyrosine should weigh 12,305 gm.

Hopkins and Savory (37) have carried out the most careful and complete investigation, thus far, of the composition of Bence-Jones' protein. They studied protein derived from three different cases. They did not succeed in crystallizing the material,

but both the physicochemical and the analytical evidence suggested the identity of the protein derived from the different sources, and the elementary compositions of all these were in excellent agreement. All three proteins contained 1.18 per cent of sulfur; thus the smallest weight of these proteins that could contain 1 atom of sulfur was 2,718 gm. Four times this value leads to 10,872 gm. and five times, to 13,590 gm. Neither result is in good agreement with the minimal molecular weight estimated from the tryptophane and tyrosine contents. If it be assumed that the molecule contains 2 molecules of tryptophane, 9 atoms of sulfur, and 10 molecules of tyrosine, the respective calculations yield 24,444, 24,462, and 24,610 gm.

Hopkins and Savory have also determined the amide nitrogen in all these cases, and found 1.30, 1.30, and 1.29 per cent. Taking 1.3 per cent as the average, the weight of protein containing 1 atom of amide nitrogen becomes 1,077. Twelve times 1,077 equals 12,924, a result that is not in good agreement with the molecular weight estimated from the tryptophane above. Multiplied by 23, the weight containing 1 atom of amide nitrogen becomes 24,771. This result is in somewhat better agreement with the weight of a molecule containing 2 molecules of tryptophane.

The amino acids were estimated in two of the three cases studied by Hopkins and Savory. Considering only those amino acids whose yields were of the same order in both cases, and of these, always taking the highest yield, we have calculated the weights of Bence-Jones' protein that would contain 1 molecule of aspartic acid, proline, phenylalanine, arginine, and histidine. With the exception of the diamino acids, these results raise no new point in the discussion, excepting in so far as the proline, phenylalanine, arginine, and histidine do not lead to estimates that are very consistent with a molecular weight of 24,500.

"The estimations of diamino acids were made" by Hopkins and Savory (37) "according to the familiar method of Kossel and Kutscher, as modified by Kossel and Patten, but with a further slight modification in connection with the separation of arginine and histidine. . . . The histidine estimations agreed fairly well, considering the small amount present, and the arginine estimations agreed very exactly. In the lysine separation however an unexplained difficulty presented itself, and" they "were unable

to make a quantitative comparison." In the following calculation we have therefore not considered Hopkins and Savory's lysine estimate. In the two cases they found 0.843 and 0.780 per cent of histidine, and 6.020 and 6.062 per cent of arginine.

More recently Lüscher, working in Hopkin's laboratory, determined the diamino acids in Bence-Jones' protein by Van Slyke's nitrogen distribution method. His determinations recalculated from nitrogen as per cent nitrogen to per cent of protein were histidine, 2.69 per cent; arginine, 4.67 per cent; and lysine, 6.79 per cent. These results do not in the least agree with those previously obtained by Hopkins. The explanation of the discrepancy is scarcely in the province of the present investigation. It might be noted, however, that the sum of the histidine and the arginine is approximately the same in both cases.

The unsatisfactory state of knowledge regarding the diamino acids in Bence-Jones' protein renders it desirable not to consider them in the present attempt to estimate its minimal molecular weight. If Hopkins' histidine is considered as correct, the minimal molecular weight of Bence-Jones' protein would be 18,398. This at first suggested to us a molecular weight of approximately 36,750, but Hopkins and Savory's own sulfur results are inconsistent with this figure and the sulfur analysis should be the more accurate. A molecular weight of 73,500 alone would allow the inclusion of the histidine and the sulfur, but such a molecular weight is probably too large for this protein. It may be added that Lüscher's results lead to no more consistent molecular weight estimates than do those of Hopkins and Savory.

Despite the discrepancies, the excellent agreement between the molecular weight calculated on the basis of Folin and Looney's tryptophane and tyrosine determinations and of Hopkins and Savory's sulfur analysis leaves little doubt that the minimal molecular weight of Bence-Jones' protein is 12,250, or twice this weight 24,500.

V.

DISCUSSION.

In the foregoing discussions, the attempt has been made to deduce the minimal molecular weights of fourteen proteins from physicochemical measurements of their equivalent combining weights, and from analytical determinations of their elementary and amino acid compositions. Although the present physicochemical and analytical evidence would appear adequate for the tentative estimation of the minimal molecular weights of these proteins, it must be admitted that in the present state of knowledge too great confidence cannot be placed upon any single determination or set of determinations. Accordingly we have believed it the province of this investigation to examine critically the results that different investigators have obtained, not the methods they have employed. The calculations have been made without attempting to evaluate the significant errors of the numerical results, in the belief that these become apparent in their comparison. Only when different methods have led different investigators to essentially the same conclusion can we proceed to the furthest implications of the result.

Occasionally there has been agreement between a number of investigators, employing different methods, whereas other workers, employing still different methods, have come to another conclusion. As an example the tryptophane content of casein may be cited. Earlier analysts succeeded in isolating as much as 1.5 per cent of this amino-acid from casein (59, 14). More recently Dakin, obtained a slightly higher yield of 1.7 per cent (16), and Folin and Looney, using their colorimetric method, estimated that 1.54 per cent was present. These results are in fair agreement with each other. Fürth and Nobel (24), using another colorimetric method, and Onslow (55), using an indirect method, believed, however, that casein contained a higher amount of tryptophane. Since Fürth and Dische (23) have now come to the conclusion that Fürth's earlier high results with Nobel were in error, it seems best to accept the relatively consistent results that indicated a tryptophane content of approximately 1.6 per cent. Meanwhile, May and Rose (52) and Jones, Gersdorff, and Moeller (43) have based their estimates of the trypto-

phane contents of a number of proteins on the amount in casein. May and Rose have assumed that casein contains 1.5 per cent and Jones and his coworkers 2.2 per cent. All of the results of these investigators are, therefore, subject to revision.

Another case in point is the histidine content of casein. Here the earlier gravimetric analysis is in good agreement with Van Slyke's estimate (71) on the basis of his nitrogen distribution method. Hanke and Koessler (30) have, however, reported a higher result on the basis of their colorimetric procedure. Without coming to any conclusion regarding the merits of the respective methods, we have felt justified in temporarily accepting the slightly lower result, dependent upon two independent investigations, as the content of histidine in casein.

To justify each decision that has been made in accepting or rejecting evidence would be to extend this discussion unduly. It seems better to indicate the guiding principles that have been employed and to leave to others, or to a later time, a still more detailed examination of evidence.

The deduction of a minimal molecular weight from physico-chemical and analytical information has not usually depended upon the accuracy of any one determination. For calculations, such as those that have been reported, yield evidence which depends not only upon the repeated determination of the same molecular component, but also upon the relations that obtain between the different components of the same molecule. When several combining and containing weights bear simple ratios to each other, their least common multiple has served as a standard in terms of which other results have been judged.

There is, of course, a danger, as there is also an advantage, in considering analytical evidence in this way. The advantage inheres in the setting up of simple, objective criteria; the danger, in the introduction of criteria that may be prejudicial to the acceptance of results that at first appear irregular. The discrepancies between the minimal molecular weights of gliadin, glutenin, and edestin estimated from Folin and Looney's determinations of cystine and tryptophane may be of this kind. From one point of view, these estimates may be considered very satisfactory, but the purposes for which they have now been employed seem to offer a new interest in the repetition and refinement of

these analyses. For if they be considered precise, the molecular weights of these proteins must be greater than 100,000.

Although it is probable, from dialysis experiments, that the molecular weights of these three proteins and also of zein, gelatin, and casein are as large as this, it is extraordinary that so many of their molecular constituents should have led to containing and combining weights that were consistent with relatively low minimal molecular weights. The cystine content of casein, which has been reported by two different investigators (21, 43), demands, as we have seen, a very high molecular weight for this protein; namely, 192,000. Very nearly all of the other amino acids have, however, been found in amounts consistent with a minimal molecular weight of 12,800. If it were established that another component did not lead to the lower figure, it would aid in fixing the true molecular weight of this protein. Similar considerations apply to the critical components of other proteins. One cannot help wondering whether the possession by casein, gelatin, and these vegetable proteins of low minimal molecular weights, but high probable molecular weights, may not indicate the manner in which these proteins are organized.

The relation between the iron and the sulfur in the hemoglobins, and between the cystine and the sulfide sulfur in different proteins, may also have wide implications. The former ratio, as we have seen, furnishes proof of the specificity of the hemoglobins. The latter suggests that certain proteins contain other sulfide sulfur-containing bodies than cysteine or cystine. This is scarcely the place, however, in which to discuss the nature of the sulfide sulfur-containing substances in proteins.

All of the calculations that have been made, and all of the deductions that have been drawn, cannot be considered as equally well demonstrated. The evidence that has been adduced for the minimal molecular weights has, on the whole, been of three kinds; that demanded by single molecular constituents, that demanded by two or more molecular constituents, and finally that suggested by certain analyses that are as yet not sufficiently well established to give confidence in their implications. Such implications have been pointed out, not so much in the belief that they are at present well founded, as in the hope that they may again be tested. The dialysis and ultrafiltration ex-

periments to be reported subsequently furnish information on so many of these points that it seems best to await the presentation of this evidence before further discussing the probable molecular weights of the proteins.

VI.

SUMMARY.

1. Solubility and electromotive force measurements have been employed in determining the equivalent combining weights for acid and for base of different proteins.

2. The equivalent combining weights of these proteins have been compared with their minimal molecular weights calculated from such elementary constituents of proteins as their iron, copper, phosphorus, sulfur, and sulfide sulfur contents, and with the minimal molecular weights calculated from the amino acids they contain.

3. The simultaneous consideration of this physicochemical and analytical information has led to the following estimates of the minimal molecular weights of fourteen proteins.

Protein.	Minimal molecular weight.
Gelatin.....	10,300
Zein.....	19,400
Gliadin.....	20,700
Hemocyanin, <i>Limulus</i>	22,700
Bence-Jones' protein.....	24,500
Edestin.....	29,000
Hemocyanin, <i>Octopus</i>	33,500
Egg albumin.....	33,800
Glutenin.....	36,300
Fibrin.....	42,000
Serum albumin.....	45,000
Hemoglobin.....	50,000
Serum globulin.....	81,000
Casein.....	192,000

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THE CARBONIC ACID-CARBONATE EQUILIBRIUM AND OTHER WEAK ACIDS IN SEA WATER.

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Sea water is a salt solution of about 3.5 per cent concentration whose various constituents are maintained in almost constant proportions. A notable exception to these constant relations occurs in the case of carbon dioxide, which is found to vary greatly in concentration. These facts of variation are undoubtedly related to the conditions of organic activity, and the concentration of carbonic acid is one determining factor for photosynthesis and an indication of energy releasing metabolic processes (Henderson, 1913; McClendon, 1918).

A pure carbonate solution is determined in its characteristics by three factors of base and hydrogen ion concentration and carbon dioxide tension. According to the work of Johnston (1916), starting with the condition

$$cP_{\text{CO}_2} = [\text{H}_2\text{CO}_3]$$

the expressions for dissociation of carbonic acid

$$[\text{H}_2\text{CO}_3] = k_1 [\text{H}^+] [\text{HCO}_3^-]$$

and

$$[\text{HCO}_3^-] = k_2 [\text{H}^+] [\text{CO}_3^{--}]$$

and substituting in the equation expressing electroneutrality

$$[\text{B}^+] + [\text{H}^+] = 2[\text{CO}_3^{--}] + [\text{HCO}_3^-] + [\text{OH}^-]$$

then

$$P_{\text{CO}_2} = \frac{[\text{H}_2\text{CO}_3]}{c} = \frac{([\text{B}^+] + [\text{H}^+]) [\text{H}^+]^2 - K_w [\text{H}^+]}{k_1 [\text{H}^+] + 2 k_1 k_2} \quad (1)$$

where P_{CO_2} is the pressure of carbon dioxide in atmospheres and c is the absorption coefficient in grams per liter divided by 22.4.

The terms $[\text{B}^+]$ and $[\text{H}^+]$ would appear easily determinable in pure carbonate solutions. Titration of sea water with acid to pH about 4.0 has been used for determining the excess base (McClendon, Gault, and Mulholland, 1917). This end-point has been selected by a number of investigators because it marks the limit for carbonic acid to exist in any form at ordinary tensions. The quantity of acid required appears to rest regularly around 25 ml. of 0.01 N acid per 100 ml. of sea water. At Tortugas, McClendon found the number about 24, off the south coast of England, Atkins (1922) found 25, and for Pacific Coast sea water in these experiments, I have found it about 24.

This titration shows the quantity of all of the weak acids combined with strong bases in sea water. Carbonates constitute about half of these weak acids, but direct evidence of the normal condition of the others is difficult to attain. By comparison of sea water titration figures with those for pure carbonate solutions evidence is presented here on: (1) the nature of sea water as a carbonate solution, and (2) the part occupied by other weak acids in the equilibrium.

EXPERIMENTAL METHODS.

It was found that the addition of hydrochloric acid in quantities less than 0.0025 M in added acid produced changes in pH which only slowly became permanent when exposed to air. Therefore, in order to prepare samples of sea water whose pH was determined and constant at the start, the acid was added and the sample aerated in a Pyrex flask for a number of hours. The fact that 12 hours of constant bubbling with air were often required before constant pH readings were reached shows how slowly equilibrium is attained in such a mixture.

For the electrometric titrations the sea water samples were treated as follows: Standard HCl was prepared by the convenient distillation method of Hulett and Bonner (1909) and checked through NaOH standardized against benzoic acid obtained from the Bureau of Standards. Appropriate quantities of this standard HCl were added to sea water, and the pH determined in a Clark electrode vessel, using a Leeds and Northrup hydrogen ion potentiometer and Eppley standard cell, with 0.1 N calomel electrode,

and saturated KCl agar liquid junctions as suggested by Schmidt and Hoagland (1919). Temperature was controlled in an air bath with a maximum deviation from 25°C. of 0.5°. Many determinations were carried out in duplicate, using two vessels and electrodes simultaneously.

Hydrogen-carbon dioxide mixtures were made in a tonometer of about 35 liters capacity, using acidified water covered with neutral paraffin oil. After partly filling the tonometer with hydrogen, the required amount of carbon dioxide was introduced and washed in with more hydrogen. Analyses were made of the mixture, using a Hempel absorption pipette and a calibrated gas pipette with mercury. Consistent agreement of duplicate determinations was obtainable to within 5 per cent for gas mixtures of 5 parts of carbon dioxide per 10,000.

Hydrogen was generated electrolytically by the electrolysis of 10 per cent sodium hydroxide solution with nickel electrodes. It was then passed over a tube of concentrated potassium hydroxide similar to that described by Clark (1920). To remove possible residual oxygen, the hydrogen was then passed through a tube of asbestos wool containing a palladium deposit and heated to about 150° by a nichrome wire coil. The hydrogen was then passed through moist glass wool in order to resaturate it with water vapor. On account of the possibility of contamination of the gas mixture by oxygen during the time of hydrogen production, a second tube of palladized asbestos was placed between the tonometer and electrode vessel.

Carbon dioxide might interfere with the electrode measurements by reducing the hydrogen pressure. Each gas mixture used was consequently standardized against 0.05 M potassium hydrogen phthalate, and no departures were found from the potential with pure hydrogen.

DISCUSSION.

Titration with $P_{\text{CO}_2} = 0$.

Fig. 1 shows the curves representing two sea water titrations and the typical titration or dilution curve for a strong base. The sea water curve was obtained from titration of sea water, aerated and shaken with hydrogen until a constant pH was reached, and then titrated with standard HCl. The second sea water sample was first acidified with HCl, aerated with hydrogen, and then titrated back with standard CO₂-free NaOH, giving the points represented by +.

In considering the results of the experiments represented in Fig. 1, it is seen that the curves generally coincide in the acid range. This would be expected, for the sea water buffers in these ranges are not effective and would have a similar influence with each method. The important fact bearing on titration to deter-

mine the "excess base" is seen in the sharp upward turn of all curves at pH 4. This is about the usually accepted end-point for the determination of base combined with carbonates, but the method is obviously open to errors unless the definite proper end-point can be accurately determined.¹

The direction taken by the curves on the alkaline side has a particular biological interest. A solution of strong base alone would have a pH of about 11.8 in 0.0024 N concentration, but sea water cannot be titrated to such an alkaline end-point with-

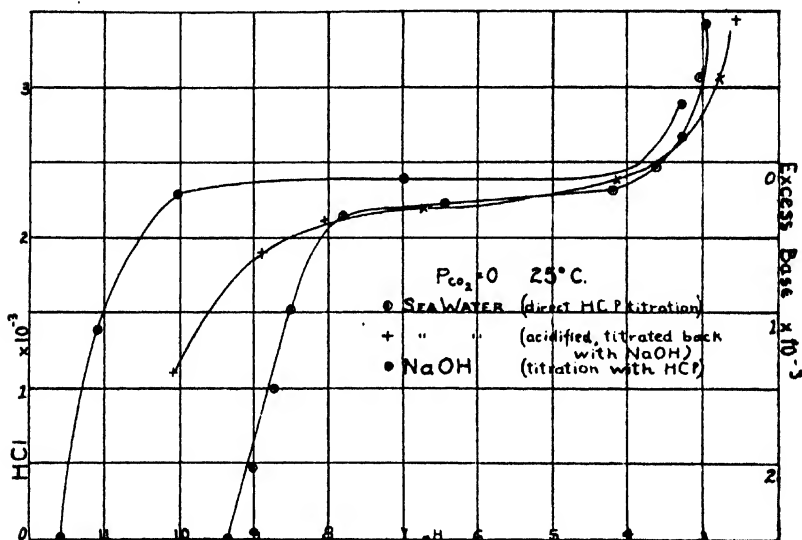


FIG. 1.

out precipitation of calcium and magnesium (Hildebrand, 1913) and consequent disturbance of the equilibrium. When the acidified sea water was titrated back with CO_2 -free alkali, it reached a pH of 9.0 at base concentration 5×10^{-4} , while the concentration of strong base alone at such a pH would be only about 10^{-5} N. The area between the two curves, then, represents the buffering of sea water by buffers which are not expelled by acidification and aeration. These are said to be principally borates, phosphates, and silicates.²

¹ Johnston, 1916, p. 958.

² McClendon, Gault, and Mulholland, 1917, p. 27.

The curve for direct titration with HCl coincides very closely with the two others in the range below the neutral point. Above, however, still another buffer effect is manifest, which must be attributed principally to residual carbonates. From a consideration of Johnston's basic formula expressing the conditions in a carbonate solution, it appears that the existence of carbonate and bicarbonate ions in a solution theoretically requires a real pressure of carbon dioxide. If this is so, the aeration with pure hydrogen would be expected to remove all of the carbon dioxide and consequently carbonate ions, and the solution should be equivalent to the sea water first acidified and titrated back with CO_2 -free base. But the slope of the curve is quite different. Sørensen³ says that the pH of solutions containing CO_2 cannot be measured with the hydrogen electrode unless they are more acid than carbonic acid or so alkaline that all of the CO_2 is bound as carbonate. He determined and found a constant pH for 0.05 M Na_2CO_3 , but stated that 0.1 M NaHCO_3 when bubbled with hydrogen went from pH 8.22 to 9.19 in about 5 hours. McClendon, Gault, and Mulholland⁴ attained a pH of 9.18 on bubbling sea water with hydrogen. They consider that the CO_2 remaining is still sufficient to convert most of the excess base to carbonate. From the curves presented here, however, it appears that the difference between the titration curve for NaOH and the titration curve for sea water, first acidified and then titrated back with NaOH, represents the non-volatile buffers; the difference between this latter curve and the curve for direct titration of aerated sea water represents the effect of unremoved CO_2 .

This alkaline end-point is related to the conditions limiting photosynthesis, for Atkins (1922), has found that marine algae can render sea water more alkaline by the removal of CO_2 during active photosynthesis to a limiting pH of about 9.2 to 9.3 at 25°. Persistent aeration with hydrogen and active photosynthesis, then, can reduce the hydrogen ion concentration of sea water to about the same limits. *Ulva*, however, was found capable of rendering sea water as alkaline as pH 9.9. Even in 1904, when the means for following such changes were only roughly developed, Loeb had observed that *Ulva* considerably

³ Sørensen, 1909, p. 190.

⁴ McClendon, Gault, and Mulholland, 1917, p. 50.

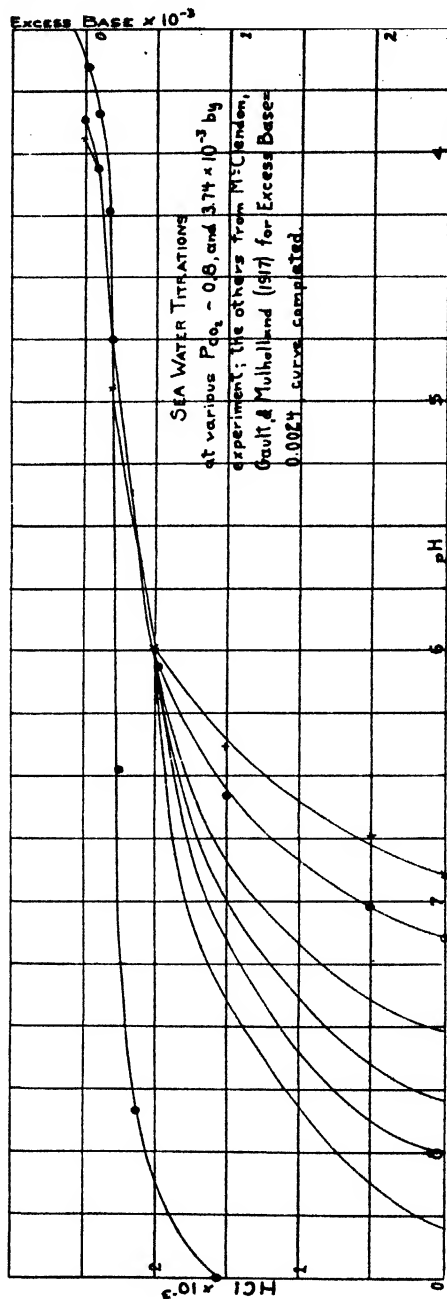


FIG. 2

increased the alkalinity of sea water during photosynthesis. When *Ulva* was kept in the dark and in artificial sea water containing no carbonates it still increased the alkalinity. In explanation he suggested that the alga gave out alkali, a process analogous to the secretion of carbonates by land plants. Bubbling with hydrogen and photosynthesis result in approximately the same final hydrogen ion concentration, one where, according to the

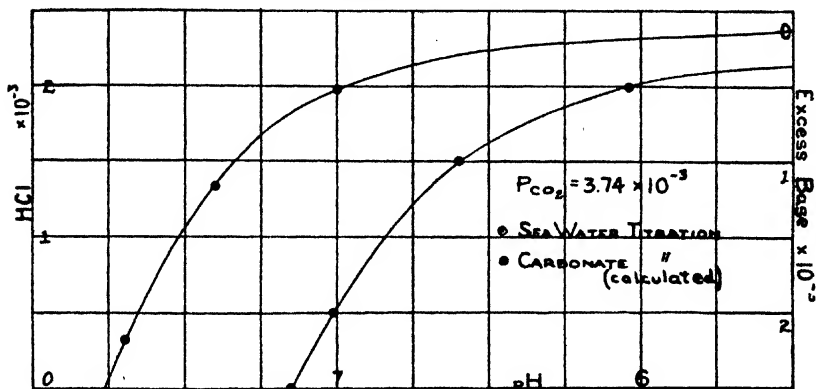


FIG. 3.

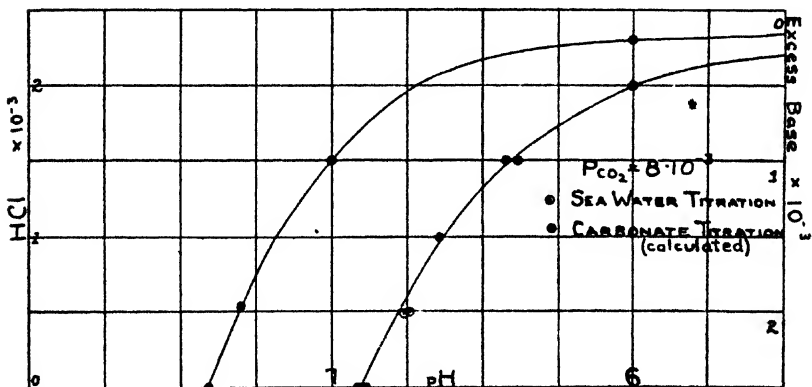


FIG. 4.

conditions, some carbonate, but no bicarbonate, still remains. Hydrogen ion concentration may be the limiting factor in photosynthesis, but it is a point which might suggest profitable evidence on the nature of the carbonate substance actually absorbed by the plant.

Titrations at Other Carbon Dioxide Tensions.

Fig. 2 shows the considerable difference between the acid titration curves of sea water at various carbon dioxide tensions. This suggests the reason for the apparent paradoxical condition shown in titrations of natural waters, where occasionally a sample will have a higher excess base concentration than another which is initially more alkaline.

Figs. 3 and 4 compare the titration values calculated from Johnston's formula for typical carbonate solutions with those found for sea water of corresponding excess base and carbon dioxide tension. There is to be seen a conspicuous difference,

TABLE I.

Comparison of the pH Found for Normal Sea Water at Different CO₂ Tensions with Those Found by McClendon, Gault, and Mulholland and Henderson and Cohn

Pco ₂	pH		
	Irving.	McClendon, Gault, and Mulholland.	Henderson and Cohn.
8×10^{-3}	6.90	6.95	6.85
3.74×10^{-3}	7.14	7.28	7.2

so that it is apparent that the conditions may not be predicted from the carbonate formula. McClendon, Gault, and Mulholland⁵ show values for the pH of normal sea water from Tortugas at different carbon dioxide tensions. Comparing their figures and those of Henderson and Cohn⁶ at 20° with mine (as in Table I) shows a close agreement.

The calculated curves given are from substitutions in equation (1), with values taken as follows, all at 25°.

$$\begin{aligned}
 k_1 &= 3.5 \times 10^{-7} \text{ (Lewis and Randall, 1923)}. \\
 k_2 &= 5.4 \times 10^{-11} \text{ (" " " 1923)}. \\
 2k_1k_2 &= 3.78 \times 10^{-17} \text{ (" " " 1923)}. \\
 K_w &= 1 \times 10^{-14} \text{ (" " " 1923)}.
 \end{aligned}$$

⁵ McClendon, Gault, and Mulholland, 1917, p. 36.

⁶ Henderson and Cohn, 1916, p. 619.

⁷ Lewis and Randall, 1923, p. 311.

⁸ Lewis and Randall, 1923, p. 312.

⁹ Lewis and Randall, 1923, p. 486.

$c = 0.03$ for a $0.5 \text{ } N$ NaCl solution (Bohr¹⁰).

$[\text{B}^+]$ is taken as 0.9 times the excess base, less the HCl added.

Other Acids than Carbonic in Sea Water and Their Dissociation Constants.

The difference between the calculated and determined excess base represents the part combined with non-volatile acid. Henderson and Cohn's artificial sea water was equivalent to the natural in buffer effect. It was $0.0022 \text{ } M$ in (excess) base and $0.0015 \text{ } M$ in boric acid, with various CO_2 pressures. According to the analyses quoted by Vernadsky¹¹ sea water is $0.0011 \text{ } M$ in silicon, $0.00018 \text{ } M$ in boron, and $0.000032 \text{ } M$ in phosphorus. These give a combined concentration of the three weak acid-forming elements of $0.0013 \text{ } M$. The condition of silicic acid and its salts can hardly be suggested with our present analytical figures and constants, and so it is impossible to determine its precise influence. But a series of comparisons may indicate more exactly the effect of these acids.

If the non-volatile acid in sea water is HA and the excess base B , then

$$[\text{HA}] = \frac{[\text{H}^+][\text{A}^-]}{k}$$

As practically all of the anions of a weak acid are furnished by its salts,

$$\frac{[\text{A}^-]}{0.9} = [\text{BA}]$$

and

$$[\text{BA}] = [\text{B}] - [\text{BC}]$$

where $[\text{BC}]$ is the calculated base in a carbonate salt solution of the same pH, and $[\text{B}]$ is the total excess base ($0.0024 \text{ } M$ in OH^-), less acid added.

Therefore

$$[\text{HA}] k = [\text{H}^+] \times 0.9 [\text{BA}]$$

and $[\text{HA}] k$ may be calculated from the data. For certain points on Figs. 3 and 4 these values are given in Table II.

¹⁰ Bohr, cited from Johnston, 1916, p. 951.

¹¹ Vernadsky, 1924, p. 8.

If the other acids than carbonic in sea water are quite non-volatile, like silicic and boric, and if they are not altered by these changes, the free acid and that combined with base will have a constant sum under all conditions. Therefore, at two different acidities, 1 and 2

$$\frac{[\text{HA}_1]}{[\text{BA}_1]} k + [\text{BA}_1] = \frac{[\text{HA}_2]}{[\text{BA}_2]} k + [\text{BA}_2]$$

and

$$k = \frac{[\text{HA}_1] k - [\text{HA}_2] k}{[\text{BA}_2] - [\text{BA}_1]}$$

Taking pairs from each figure, it is seen that k does have a value constant at $n \times 10^{-8}$, as far as the limits of experimental accuracy

TABLE II.

Calculations for the Dissociation Constants of Acids Other than Carbonic in Sea Water.

	No.	pH	[BA]	[HA] k	k
$P_{\text{CO}_2} = 3.7 \times 10^{-3}$	1	7.0	1.4×10^{-3}	1.26×10^{-10}	11.7×10^{-8} (1-2)
	2	6.0	0.35×10^{-3}	3.2×10^{-10}	30×10^{-8} (2-3)
	3	6.7	0.85×10^{-3}	1.53×10^{-10}	2×10^{-8} (1-3)
$P_{\text{CO}_2} = 8 \times 10^{-3}$	1	6.7	1.14×10^{-3}	2.0×10^{-10}	8×10^{-8} (1-2)
	2	6.4	0.66×10^{-3}	2.4×10^{-10}	6×10^{-8} (2-3)
$P_{\text{CO}_2} = 0$	1	7.0	0.18×10^{-3}	1.62×10^{-11}	9×10^{-7} (1-2)
	2	6.0	0.11×10^{-3}	0.99×10^{-10}	

and these methods of calculation suggest. Calculations from pairs 1 to 3 are unsatisfactory because the points compared are too close and on unfavorable slopes for comparison.

Even calculating for P_{CO_2} equals 0, k has a value in general agreement with the others at pH 6 and 7. In the more alkaline range of this curve, the values of k , however, would be calculated as far too small. If the real value of k is constant, as is to be expected, the low value calculated suggests a much higher concentration of the free acid HA than can really exist in the solution. The behavior of silicates, especially in the presence of calcium and magnesium, is an uncertain factor, but it is probable that there is still some residual carbonic acid, as was suggested by the titration back with alkali of sea water first acidified (Fig. 1).

This accounts for the apparent high concentration of these other still weaker acids.

Acid Limits of CO₂ Solubility.

The acid end-point of carbonate titrations is usually taken as at pH about 4.0. At this pH, in a pure carbonic acid solution, the CO₂ tension would approach 1 atmosphere. In any carbonate solution, the equation shows that CO₂ tension is a function of both the base and pH. A sodium acetate-acetic acid buffer solution was prepared with a pH of 4.46. This was then bubbled with pure CO₂ and left in an open beaker. 2 hours later, the addition of saturated barium hydroxide solution formed a precipitate, indicating that some CO₂ remained in solution. When another sample was boiled and tested after cooling, it formed no precipitate.

A second sodium acetate-acetic acid buffer mixture at pH 3.8 was bubbled with CO₂ and likewise left in an open beaker. The next day addition of barium hydroxide solution formed no precipitate. As Johnston (1915) states that the solubility product constant for BaCO₃ is 7×10^{-9} , and enough barium hydroxide was added to make the solution strongly alkaline, it is clear that the discernible solubility limit for carbon dioxide is between the two acidities tried. The solubility of CO₂ is, then, extremely small unless conditions permit its ionization, and it is really the solubility of H₂CO₃ which determines the capacity of a solution for this gas.

SUMMARY.

The large amount of CO₂ in the ocean is significant to the fixation and use of energy by organisms. It occurs as H₂CO₃, HCO₃⁻, and CO₃⁼, the quantities of each depending upon the pH and excess base. Titration curves at different CO₂ tensions differ in a regular manner. They also vary considerably from titration curves for pure carbonate solutions. This difference is assignable to the fact that sea water contains other weak acids, whose amount and behavior is not yet known. Near the neutral range, however, these other acids together produce an effect corresponding to an acid with dissociation constant $k = n \times 10^{-9}$.

Because it is a mixture of weak acids and their salts, and because

some of the acids are not volatile, the pH of sea water may be varied by the addition of acid or alkali and the change fixed at any CO_2 tension. This CO_2 tension must be established in the solution, however, by prolonged bubbling until the equilibrium is reached.

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